三人行，必有我师焉：择其善者而从之，其不善者而改之

When walking in the company of other men, there must be one I can learn something from. I shall pick out his merits to follow and his shortcomings for reference to overcome my own.

——Confucius (551B.C. - 479B.C.)

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Epidemiology of *Chlamydiaceae* in livestock and emerging *Chlamydia psittaci* infections in chickens

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List of abbreviations

aMPV	Avian metapneumo virus
AT	ArrayTube™
BALT	Bronchus associated lymphoid tissue
BGM	Buffalo Green Monkey
C.	*Chlamydia*
CD	Cluster of differentiation
CFT	Complement fixation test
CI	Confidence interval
CSC	China Scholarship Council
CTC	Chlortetracycline
DIF	Direct immunofluorescence staining
DNA	Deoxyribonucleic acid
dpi	Day (s) post infection
EB	Elementary body
ELD_{50}	The 50% median egg lethal dose
ELISA	Enzyme-linked immune sorbent assay
HRM	High-resolution melting-curve
IBV	Infectious bronchitis virus
ICFT	Indirect complement fixation test
IHA	Indirect haemagglutination assay
IL	Interleukin
ILTV	Infectious laryngotracheitis virus
LAMP	Loop-mediated isothermal amplification
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MIF	Micro-immunofluorescence
MLST	Multi-locus sequence typing
MMLS	Maximum mean lesion scores

MOMP	Major outer membrane protein
MRL	Maximum residue limits
Mt	Million tons
NAAT's	Nucleic acid amplification techniques
NF	Nuclear factor
NI	Normalized signal intensity
OEA	Ovine enzootic abortion
OIE	World Organisation for Animal Health
ompA	Outer membrane protein A
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PDS	Periparturientdysgalactiae syndrome
POMP	Polymorphic outer membrane protein
PPA	Peroxidase conjugated *Staphylococcal* protein A
RB	Reticulate body
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SPF	Specific-pathogen-free
STD	Standard
$TCID_{50}$	The 50% tissue culture infective dose
TGF	Transforming growth factor
TH1	T helper cells
TWAR	Taiwan Acute Respiratory

Study objectives

The first part of the present study aims to review the epidemiological status of *Chlamydiaceae* infections in Chinese livestock in order to make a comparison with the European situation. In addition, it was our purpose to study the epidemiology of *Chlamydia (C.) abortus* in Belgian ruminants, as the occurrence of this important zoonotic pathogen has not been examined in Belgium. We also wanted to perform an epidemiological study on *C. psittaci* infections in Belgian and French broiler chickens, as limited data are available.

We found that *Chlamydia abortus* infections were less common in Belgian ruminants, as compared to other countries. Surprisingly, our data showed the emergence of *C. psittaci* infections in broilers. However, we still had to prove the clinical importance of this finding. That is why we subsequently focused on studying the pathology of *C. psittaci* outer membrane protein A (*ompA*) genotypes, originating from different geographical areas and bird species, for specific-pathogen-free (SPF) chickens.

Chlamydiaceae is a family of closely related obligate intracellular Gram-negative bacteria causing a wide range of disease in both humans and animals. *Chlamydiaceae* all share a distinctive biphasic developmental cycle comprising of two forms, an extracellular, non-multiplying infectious form known as an elementary body (EB) and an intracellular, multiplying non-infectious body known as a reticulate body (RB). Well-known zoonotic *Chlamydiaceae* in livestock include *C. abortus* and *C. psittaci*.

Worldwide, *Chlamydiaceae* infections cause important economic losses to the livestock industry. Their occurrence and clinical impact is well documented in the English literature. However, data on the epidemiology of chlamydiosis in China are scarcely represented in the English literature. In view of China's tremendous increase in poultry and livestock production and China's share on the global market over the last decade, **we therefore started this study by** reviewing the English and Chinese literature on *Chlamydiaceae* infections in Chinese livestock. Data will be used to make a comparison between the Chinese and European situation.

C. abortus is a major abortigenic agent in ruminants, causing ovine enzootic abortion (OEA) in sheep. Chlamydial abortion in sheep has a worldwide prevalence, with the exception of Australia and New Zealand. Although OEA is a reproductive disease, the principal route of transmission to naive sheep is thought to be via an oro-nasal route, most likely from heavily infected placentas from ewes that have aborted and contaminate the environment. *C. abortus* is a well-recognized and potentially fatal zoonosis, presenting a major hazard to pregnant women who contact with livestock, particularly at lambing. The occurrence of *C. abortus* is intensively monitored in the major sheep-producing countries, such as the UK, as besides economic losses *C. abortus* poses a threat to public health. However, epidemiological data on *C. abortus* infections in Belgian ruminants are lacking. Therefore, **the second aim** of this study was to examine the prevalence of *C. abortus* in Belgian sheep, goat and cattle herds.

C. psittaci causes chlamydiosis in birds, which is a respiratory infection leading to systemic bacterial dissemination and potentially mortality. Zoonotic transfer occurs by inhalation and may pass subclinical or manifest as a flu-like illness to a potentially fatal systemic disease. *C psittaci* includes 7 known avian *ompA* genotypes designated A to F and E/B (Everett *et al*., 1999a; Geens *et al*., 2005a). Some avian genotypes appear to occur more often in a specific order of birds. Genotype A for instance, is endemic among psittacine birds. Genotype B is endemic in pigeons. Waterfowl, most frequently seem to be infected with genotype C strains while genotype D strains are often associated with turkeys. However, genotype E, also known as Cal-10, MP, or MN was first isolated during an outbreak of pneumonia in humans during the early 1930s. Later on, genotype E isolates were obtained from a variety of bird species including ducks, pigeons, ostriches and rheas. Genotype F is represented by the psittacine isolates VS225, Prk Daruma and 10433-MA, but has also been isolated on a Belgian turkey farm (Van Loock *et al*., 2005a).

Limited epidemiological data on *C. psittaci* infections in chickens from 1960 to 2000 suggest that chickens are relatively resistant to disease. However, scientific evidence on the virulence or a-virulence of *Chlamydia* in chickens is scarce, as chicken isolates are rare. Up to date, *C. psittaci* genotypes B and D were most frequently found in broilers (Vanrompay *et al*., 1997; Dickx *et al*., 2011). Beeckman *et al*. (2010) already performed a study in chicken macrophages

(HD11 cells) comparing host pathogen interactions of the low virulent genotype B reference strain CP3 (Bankowski and Page, 1959; Piraino, 1969) to the highly virulent genotype D strain (92/1293). CP3 was isolated in 1957 from a Californian pigeon while 92/1293 was isolated in 1992 from Dutch diseased turkeys (Vanrompay *et al*., 1993). The genotype D strain: 1) clearly induced actin recruitment to the site of *Chlamydia* entry and invaded the host cells more efficiently, 2) initiated host cell degeneration at earlier time points, and 3) survived and proliferated better in macrophages when compared to the low virulent CP3 strain. **The third aim** of our study was to study the pathogenicity of CP3 and 92/1293 *in vivo* in SPF chickens and to compare the results with the previously obtained *in vitro* (HD11 cells) data.

Recently, *Chlamydia* outbroke in French broilers, linked with pneumonia in poultry workers (Laroucau *et al*., 2009). **The fourth aim** of this study, was gathering information on the current epidemiological status of *C. psittaci* infections in broilers raised in Northern France and Belgium and additionally, to fullfill Hill-Evans postulates for chicken infectious *C. psittaci* strains in order to prove the virulence of *C. psittaci* for chickens.

Chapter One

Chlamydiaceae infections in Chinese livestock

Part A

Diagnosis, clinical disease, prevention and treatment of *Chlamydiaceae* infections in Chinese livestock

Adapted from:

Lizi Yin[#], Isabelle Kalmar[#], Jeanne Boden and Daisy Vanrompay. Chlamydial infections in Chinese livestock. OIE Scientific and Technical Review. (accepted)

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Abstract

The occurrence and impact of chlamydial infections in Western livestock is well documented in the international literature. In contrast, we know less on *Chlamydiaceae* infections in Chinese livestock. Yet, China's livestock production and its share on the global market have increased significantly over the last decade. Here we review the relevant English and Chinese literature on the epidemiology of *Chlamydiaceae* infections in Chinese livestock. This paper further discusses biosecurity measures, prophylaxis and treatment of *Chlamydiaceae* infections in China's livestock production as compared to Western practices. Chlamydial infections in Chinese livestock are highly prevalent and cause important economic losses as in the rest of the world. Surveillance data and diagnostic results of abortion outbreaks in cattle, sheep and goat evidence the importance of virulent chlamydial infections in China's major ruminant species in at least 20, 12 and 10 of China's 32 provinces, autonomous regions and municipalities, respectively. Data of 23 of China's provincial divisions also indicate its widespread presence in industrial produced swine across China. Less is known on *Chlamydiaceae* infections in yak, buffalo and horses, but available data indicate a high prevalence in China's populations. In these reports, chlamydiosis was related to abortion in yak and pneumonia in horses. In Western countries, livestock *Chlamydiales*, as *Chlamydiaceae* infections in general, are principally treated with antibiotics. In China, however, herbal medicine is often used in conjunction with antibiotic treatment or applied as an alternative hereto.

Keywords: *Chlamydiaceae*, livestock, diagnosis, biosecurity, antibiotic treatment, herbal medicine, China, zoonosis, review

1. Introduction

Chlamydiaceae are Gram-negative, obligate intracellular bacteria with a unique biphasic development cycle. The pathogen differentiates intracellularly between the elementary body (EB) and the reticulate body (RB). The EB's are the extracellular infectious form, whereas RB's are the non-infectious form only present inside the living cell. Zoonotic transmission occurs by inhalation or accidental ingestion of EB's from animal excretions and discharges.

In the following, we consider chlamydial infections in livestock. As defined here, this refers to ruminants, pigs and equines raised in agricultural settings, and does not include poultry, rabbits, game or farmed fish. Livestock *Chlamydiaceae* include *Chlamydia (C.) abortus, C. pecorum, C. suis, C. psittaci* and equine *C. pneumoniae. Chlamydia abortus* is a major abortigenic agent in ruminants and causes abortion in pregnant women upon zoonotic transmission (Aitken, 2000). Infection in swine and horses may also result in abortion, but does not result in abortion storms as seen in affected sheep or goat flocks (Aitken, 2000; Everett, 200). *Chlamydia pecorum* infections in ruminants, swine and horses have been associated with urogenital symptoms, reproductive failure, conjunctivitis, respiratory distress, polyarthritis and pericarditis (2). Hitherto, apart from a sporadic finding in birds of prey (Lemus *et al*., 2010), *C. suis* infections have been thought to be restricted to swine. However, preliminary data indicate the possibility of zoonotic transfer (Vanrompay *et al.*, unpublished data). In swine, virulent *C. suis* strains may cause reproductive failure, and respiratory and intestinal signs (Schautteet and Vanrompay, 2011). The zoonotic chlamydial species *C. psittaci*, of which birds are the natural host, also infects pigs and cattle (Eggemann *et al*., 2000; Vanrompay *et al*., 2004; Reinhold *et al*., 2011). Equine *C. pneumonia* is currently confined to one respiratory isolate, for which experimental infection in horses remains asymptomatic (Mair and Wills, 1992).

2. Global importance of Chinese livestock production

China's changing political orientation from 1985 onwards, facilitated its agricultural growth rate (Windhorst, 2008). Accordingly, China has become a major livestock producer, exporting meat and dairy products globally. Major destinations of export are within the Asian region, in

particular, Japan, Kirghizstan, Jordan and the United Arab Emirates. China's most important meat export product is pork, wherein it represents the fifth largest exporting country worldwide. According to FAOSTAT 2010 data, China's total annual meat production comprises 80.74 Mt, of which 64.0% pork, 13.0% ruminant meat and 0.5% equine meat. This represents 47.3%, 13.2% and 43.7% of the global pork, ruminant meat and equine meat production. Meat of sheep, goat and buffalo, which accounts for merely 2.6%, 2.3% and 0.4% of China's total meat production, contributes for 24.3%, 36.2% and 9.1% of global production respectively. Equine meat production in China is represented by 46.8% horse, 40.1% ass, and 13.0% mule meat, accounting for 27.4%, 90.1% and 98.8% of global production, respectively (FAOSTAT, 2010).

In 2000, China's share of global milk production was merely 2.1%. However, during the past decade, China's explosive increase in dairy production (+232.6%) resulted in a current global share of 5.7% and amounts 41.15 Mt annually. Sheep and goat milk represent only 2.3% and 1.4% of dairy products globally, of which China produces only a minor fraction. The same accounts for buffalo and camel milk. Yet, with only 3.4% of global buffalo milk production, China is considered the third largest producer worldwide after India and Pakistan (FAOSTAT, 2010). Finally, although not included in FAOSTAT statistics, yaks comprise a notable ruminant species in China. Its population is estimated at 13 million animals, representing 92.8% of the global population (Huang, 1996). These sturdy, but low-milk yielding ruminants are perfectly adapted to the high altitude and related extreme environmental conditions of the Qinghai-Tibet Plateau (Tibetan Autonomous Region, Qinghai, Gansu, Sichuan and Yunnan), such as low temperature, low oxygen and high solar radiation (Zhaoli *et al*., 2002; Dong *et al*., 2007).

3. Diagnosis of *Chlamydiaceae* in Chinese livestock

3.1. Seroprevalence

The majority of serological data on *Chlamydiaceae* in Chinese livestock is obtained by commercially available indirect haemagglutination assay (IHA) developed at the Lanzhou Veterinary Research Institute. The Lanzhou IHA kit uses antigen prepared from the Chinese ovine *C. abortus* strain B11001. Only a few reports make use of the standard OIE recommended

complement fixation test (CFT) or enzyme-linked immunosorbent assays (ELISA) for detecting chlamydial antibodies. The vast amount of serological data on ruminants demonstrates that *Chlamydiaceae* are endemic in small and large ruminants across China, with the exception of Jiangsu, Shanghai and Shaanxi (Tables 1 and 2). Similarly, prevalence data of 23 of China's 32 provinces, autonomous regions and municipalities demonstrate that *Chlamydiaceae* infections are widespread in industrial produced swine across China (Table 3). Seroprevalence data on horses are limited to four Chinese provinces, but as well indicate presence of chlamydial antibodies (Table 4).

Table 1. Prevalence (%) of chlamydial antibodies in Chinese sheep and goats.

Year	Province	Seroprevalence	Methods	Species	Symptoms	Reference
1981	Qinghai	27.7 (47/176)	CFT	goats	NA	(Shuai *et al*., 1981)
1981	Gansu	6.8 (4/11)	CFT	goats	NA	(Shuai *et al*., 1981)
1983	Xinjiang	25.5 (27/106)	CFT	sheep/goats	abortion	(Wang *et al*., 1986)
1985	Hubei	6.8 (324/4753)	CFT/IHA	sheep/goats	NA	(Yang *et al*., 1992)
1985	Hubei	29.8 (25/84)	CFT/IHA	sheep/goats	abortion, conjunctivitis	(Yang *et al*., 1992)
1986	Yunnan	60.7 (17/28)	ELISA[a]	goats	abortion	(Jin *et al*., 2005)
1986	Xinjiang	20.0 (14/70)	ELISA[a]	goats	abortion	(Jin *et al*., 2005)
1989	Qinghai	15.4 (53/344)	IHA	sheep/goats	NA	(Han *et al*., 2010)
1989	Jilin	6.0 (138/2291)	CFT	sheep	NA	(Liang and Xiao, 1990)
1990	Zhejiang	3.2 (28/872)	IHA	sheep	NA	(Wang and Zhao, 1990)
1991	Gansu	6.6 (13/198)	IHA	sheep	NA	(Liu *et al*., 1991)
1991	Shaanxi	1.6 (103/6615)	IHA	sheep/goats	NA	(Lin *et al*., 1991)
1991	Gansu	34.4 (360/1047)	IHA	sheep/goats	abortion	(Du *et al*., 1991)
1993	Xinjiang	8.48 (126/1486)	IHA	sheep	NA	(Jin *et al*., 1993)
1993	Xinjiang	27.1 (23/85)	IHA	sheep	abortion	(Jin *et al*., 1993)
93-95	Taiwan	82.0 (246/300)	ELISA[b]	goat	abortion	(Liao *et al*., 1997)
97-02	Beijing	21.0 (713/3398)	IHA	sheep	NA	(Feng *et al*., 2007)
1998	Hunan	5.9 (29/496)	IHA	goats	NA	(Qiu *et al*., 1998)
99-00	Taiwan	16.7 (4/24)	ELISA[b]	goats	healthy	(Wang *et al*., 2001)
99-00	Taiwan	58.0 (52/112)	ELISA[b]	goats	aborted	(Wang *et al*., 2001)
2000	Yunnan	26.1 (1024/3917)	IHA	sheep/goats	NA	(Wang *et al*., 2000)

Table 1-continued. Prevalence (%) of chlamydial antibodies in Chinese sheep and goats.

Year	Province	Seroprevalence	Methods	Species	Symptoms	Reference
2000	Guangxi	2.0 (43/2168)	IHA	goats	NA	(Wu *et al*., 2000)
2001	Qinghai	2.9 (20/686)	IHA	goats	NA	(Han, 2001)
2001	Guangxi	1.1 (69/6273)	IHA	goats	NA	(Huang *et al*., 2001)
2003	Ningxia	19.3 (113/586)	IHA	sheep	cough, wheezing, thin	(Liu *et al*., 2003)
2003	Yunnan	32.3 (1268/3925)	IHA	goats	NA	(Liu *et al*., 2003)
2004	Qinghai	11.2 (20/179)	IHA	goats	NA	(Yang, 2004)
2005	Qinghai	6.7 (8/165)	IHA	sheep	NA	(Lu *et al*., 2005)
2006	Sichuan	5.6 (15/268)	IHA	sheep	NA	(Qiu *et al*., 2006)
2007	Beijing	18.0 (116/646)	IHA	sheep	NA	(Feng *et al*., 2007)
2007	Henan	18.4 (7/38)	IHA	goats	abortion	(Yao *et al*., 2008)
2007	Qinghai	4.9 (11/224)	IHA	sheep	NA	(Yuan and Ma, 2007)
2008	Qinghai	5.5 (13/237)	IHA	sheep	NA	(Fan *et al*., 2008)
2009	Gansu	26.3 (172/654)	IHA	sheep	abortion, orchitis, arthritis	(Wang and Wei, 2009)
2010	Qinghai	2.6 (8/303)	IHA	sheep/goats	NA	(Han and Chen, 2010)
2010	Qinghai	7.4 (91/1223)	IHA	sheep	NA	(He *et al*., 2010)
2010	Qinghai	19.0 (33/174)	IHA	goats	abortion	(Ran *et al*., 2010)
2010	Qinghai	3.9 (7/179)	IHA	sheep	NA	(Ran *et al*., 2010)
2010	Qinghai	8.6 (27/314)	IHA	sheep	abortion	(Tie, 2010)
2010	Qinghai	9.2 (29/316)	IHA	sheep	NA	(Min *et al*., 2010)
2010	I.Mongolia	7.6 (103/1360)	IHA	sheep	abortion, orchitis	(Wang *et al*., 2010)
2010	Shaanxi	2.9 (21/729)	IHA	goats	NA	(Zhao *et al*., 2011)

IHA: indirect haemagglutination assay (Lanzhou Veterinary Research Institute, China);

CFT: complement fixation test (in-house developed);

ELISA: enzyme-linked immunosorbent assay ([a]in-house developed; [b]ImmunoComb);

NA: no information on health status available

Table 2. Prevalence (%) of chlamydial antibodies in Chinese large ruminants

Year	Province	Seroprevalence	Methods	Species	Symptoms	Reference
1985	Hubei	8.7 (382/4386)	CFT/IHA	cattle (dairy)	NA	(Yang *et al*., 1992)
1986	Hubei	26.8 (37/138)	CFT/IHA	cattle (dairy)	abortion, pneumonia, conjunctivitis	(Yang *et al*., 1986)
1988	Qinghai	29.0 (45/155)	CFT	yak	Abortion	(Shuai *et al*., 1988)
1989	Qinghai	26.7 (24/90)	IHA	cattle	NA	(Han and Chen, 2010)
1989	Hunan	8.1 (33/410)	IHA	cattle (dairy)	NA	(Jiang *et al*., 1989)
1991	Shaanxi	28.3 (107/378)	IHA	cattle (dairy)	NA	(Lin *et al*., 1991)
1991	Sichuan	6.5 (7/107)	IHA	cattle	NA	(Zuo *et al*., 1991)
1993	Xinjiang	4.9 (35/711)	IHA	cattle	NA	(Jin *et al*., 1993)
1994	Qinghai	42.7 (236/553)	IHA	yak	abortion	(Yuan *et al*., 1994)
1996	Xinjiang	2.1 (6/288)	IHA	yak	NA	(Wang *et al*., 1996)
1996	Xinjiang	1.8 (3/171)	IHA	cattle (dairy)	NA	(Wang *et al*., 1996)
1996	Hainan	2.9 (15/526)	IHA	yak	NA	(Liazhong, 2002)
99-00	Taiwan	51.3 (377/735)	ELISA	cattle	healthy	(Wang *et al*., 2001)
99-00	Taiwan	71.4 (45/63)	ELISA	cattle	aborted	(Wang *et al*., 2001)
2000	Shandong	16.1 (10/62)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Henan	25.7 (9/35)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Ningxia	16.8 (16/95)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Hebei	23.1 (3/13)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Shaanxi	42.2 (57/132)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Gansu	10.8 (4/37)	IHA	cattle (meat)	NA	(Zhou *et al*., 2000)
2000	Gansu	20.7 (18/87)	IHA	yak	NA	(Zhou *et al*., 2000)
2000	Gansu	25.5 (24/47)	IHA	yak	NA	(Qiu *et al*., 2001)
2000	Qinghai	15.5 (22/142)	IHA	yak	NA	(Zhou *et al*., 2000)
2000	Sichuan	25.6 (10/39)	IHA	yak	NA	(Zhou *et al*., 2000)
2000	Yunnan	34.6 (823/2378)	IHA	cattle	NA	(Wang *et al*., 2000)
2000	Guanxi	20.0 (10/50)	IHA	buffalo	NA	(Geilhausen, 2002)
2000	Anhui	18.7 (28/150)	IHA	buffalo	NA	(Geilhausen, 2002)
2000	Jiangsu	14.0 (20/143)	IHA	buffalo	NA	(Geilhausen, 2002)

Table 2-continued. Prevalence (%) of chlamydial antibodies in Chinese large ruminants

Year	Province	Seroprevalence	Methods	Species	Symptoms	Reference
02-04	Guangdong	49.1 (27/55)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Jiangsu	0.0 (0/20)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Shanghai	0.0 (0/49)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Henan	20.8 (10/48)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Ningxia	25.4 (177/698)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Gansu	43.2 (19/44)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Shaanxi	10.3 (25/243)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Shanxi	10.0 (1/10)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	I. Mongolia	15.7 (11/70)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
02-04	Heilongjiang	17.2 (20/116)	IHA	cattle (dairy)	NA	(Changqing *et al*., 2006)
2004	Qinghai	19.2 (30/156)	IHA	yak	abortion	(Ma *et al*., 2004)
2007	Ningxia	28.4 (108/380)	IHA	cattle (dairy)	abortion	(He *et al*., 2007)
2008	Ningxia	53.3 (16/30)	IHA	cattle (dairy)	abortion	(Yang *et al*., 2008)
2010	Qinghai	2.0 (6/300)	IHA	cattle	NA	(Han and Chen, 2010)
2010	Qinghai	5.9 (19/321)	IHA	yak	NA	(Tie, 2010)
2010	Qinghai	2.5 (25/1410)	IHA	cattle	NA	(He *et al*., 2010)

IHA: indirect haemagglutination assay (Lanzhou Veterinary Research Institute, China);

CFT: complement fixation test (in-house developed);

ELISA: enzyme-linked immunosorbent assay ([a]in-house developed; [b]ImmunoComb);

NA: no information on health status available

Table 3. Prevalence (%) of chlamydial antibodies in Chinese swine

Year	Province	Seroprevalence	Methods	Symptoms	Reference
1985	Hubei	29.7 (244/821)	IHA	NA	(Jiang *et al*., 1985)
1986	Qinghai	33.3 (101/333)	IHA	NA	(Qiu *et al*., 2003)
1987	Sichuan	11.5 (462/2989)	IHA	NA	(Qiu *et al*., 2003)
1987	Xinjiang	22.2 (303/1428)	IHA	NA	(Qiu *et al*., 2003)
1987	Hubei	29.9 (256/857)	ICFT	NA	(Meng *et al*., 1987)
1988	Shanxi	38.0 (19/50)	IHA	NA	(Qiu *et al*., 2003)
1989	Hunan	9.0 (10/111)	IHA	abortion, still birth	(Jiang *et al*., 1989)
1990	Guanxi	21.2 (303/1428)	IHA	NA	(Qiu *et al*., 2003)
1991	Yunnan	24.5 (1342/5477)	IHA	NA	(Qiu *et al*., 2003)
1992	Guangdong	50.2 (542/1080)	IHA	NA	(Qiu *et al*., 2003)
1992	Gansu	42.2 (1604/3709)	IHA	NA	(Qiu *et al*., 2003)
1993	Henan	35.1 (10/28)	IHA	NA	(Qiu *et al*., 2003)
1994	Guangdong	50.2 (542/1080)	IHA	NA	(Ren *et al*., 1994)
1995	Henan	29.1 (32/110)	CFT	NA	(Chen *et al*., 1995)
		47.3 (52/110)	ELISA[a]		
1999	Sichuan	80.0 (48/60)	IHA	NA	(Qiu *et al*., 2003)
1999	Ningxia	15.1 (18/119)	IHA	NA	(Qiu *et al*., 2003)
2000	Qinghai	42.9 (6/4)	IHA	NA	(Qiu *et al*., 2003)
2000	Henan	18.2 (6/33)	IHA	NA	(Qiu *et al*., 2003)
2000	Hainan	38.2 (21/55)	IHA	NA	(Qiu *et al*., 2003)
2000	Hubei	37.5 (3/8)	IHA	NA	(Qiu *et al*., 2003)
2000	Hunan	16.7 (6/36)	IHA	NA	(Qiu *et al*., 2003)
2001	Jianxi	50.0 (4/8)	IHA	NA	(Qiu *et al*., 2003)
2001	Liaoning	43.2 (32/74)	IHA	NA	(Qiu *et al*., 2003)
2001	Heilongjiang	77.1 (27/35)	IHA	NA	(Qiu *et al*., 2003)
2001	Jilin	20.5 (8/39)	IHA	NA	(Qiu *et al*., 2003)
2005	Shanghai	41.4 (65/157)	IHA	NA	(Jin *et al*., 2005)

Table 3-continued. Prevalence (%) of chlamydial antibodies in Chinese swine

Year	Province	Seroprevalence	Methods	Symptoms	Reference
2005	Jiangsu	1.7 (3/174)	IHA	NA	(Jin *et al*., 2005)
2005	Zheijiang	6.8 (12/176)	IHA	NA	(Jin *et al*., 2005)
2007	Guangxi	51.0 (197/386)	IHA	abortion	(Lu, 2007)
2007	Sichuan	21.7 (26/120)	IHA	NA	(Zhang *et al*., 2007)
		35.8 (43/120)	ELISA[a]		
2008	Fujian	27.7 (641/2,313)	IHA	NA	(Zhou *et al*., 2008)
2010	Guangdong	30.8 (313/1,017)	IHA	NA	(Xu *et al*., 2010)
2011	Yunnan	19.4 (232/1257)	IHA	NA	(Bi *et al*., 2011)
2012	Beijing	4.1 (21/507)	IHA	NA	(Tian *et al*., 2012)
		2.2 (11/507)	ELISA[b]		

IHA: indirect haemagglutination assay (Lanzhou Veterinary Research Institute, China)
CFT: indirect complement fixation test (in-house developed)
ELISA: enzyme-linked immunosorbent assay ([a]in-house developed, [b] ID Screen® *Chlamydia abortus* ELISA)

Table 4. Prevalence (%) of chlamydial antibodies in Chinese horses

Year	Province	Seroprevalence	Methods	Symptoms	Refs
1985	Hubei	9.3 (70/754)	CFT and IHA	NA	Xie and Preast, 2010
1992	Qinghai	6.9 (6/87)	IHA	pneumonia, diarrhea, swollen joints	Bao and Guo, 1992
1993	Xinjiang	2.0 (5/251)	IHA	NA	Jin *et al*., 1993
2000	Yunnan	35.4 (953/2691)	IHA	NA	Wang *et al*., 2000

IHA: indirect haemagglutination assay (Lanzhou Veterinary Research Institute, China)
CFT: complement fixation test (in-house developed)
NA: no information on health status available

Likewise IHA and CFT, ELISAs using whole chlamydial organisms as target antigen make no distinction between chlamydial species, as the lipopolysaccharides (LPS) of *Chlamydiaceae* are family specific. Two studies on Chinese swine of unknown status compared these serological

methods. Chen *et al.*, (1995) prepared a Dot-ELISA and CFT using the German ovine abortion strain B394, results revealing seropositivity rates of 47.3% (52/110) and 29.1% (32/110), respectively (Chen *et al*., 1995). In a similar trial, a comparison was made between the widely used Lanzhou IHA kit and an in-house Dot-PPA-ELISA based on strain B394. The highest seroprevalence rates were recorded with Dot-PPA-ELISA: 35.8% (43/120) compared to 21.7% (26/120) with IHA (Zhang *et al*., 2007). Both authors concluded highest sensitivity using respective ELISA's. However, each of these tests is prone to false positives resulting from cross-reactivity of target antigen with antibodies against, for instance, LPS of other Gram-negative bacteria (Brade *et al*., 1987). This is indicated by comparative results of the Lanzhou IHA kit and the ID Screen® *Chlamydia abortus* indirect ELISA (ID-VET Innovative Diagnostics, Montpellier, France) that uses a synthetic *C. abortus* specific fragment of the major outer membrane protein (MOMP). Results revealed an almost twofold seropositivity rate when using the IHA kit (4.14%; 21/507) compared to the ID-VET ELISA (2.17%; 11/507) (Tian *et al.*, 2012). At present, superior sensitivity and specificity for species specific detection of *C. abortus* antibodies is proposed to be an ELISA using recombinant protein fragments of the *C. abortus* polymorphic outer membrane protein POMP90 (Wilson *et al*., 2009). Chinese researchers are currently also focusing on ELISAs using recombinant fragments of the *C. abortus* POMPs and MOMP VDI and/or II, as those sequences revealed a great deal of diversity between *C. abortus* and *C. pecorum* strains. However, to our knowledge, such tests have not yet been applied to livestock.

3.2. Culture, antigen and gene detection in Chinese livestock

As in the rest of the world, diagnosis of ongoing *Chlamydiaceae* infections in Chinese livestock was initially performed by culture, for which Chinese laboratories essentially use yolk sac inoculation of 6 to 7-day-old specific-pathogen-free (SPF) chicken embryonated eggs (Table 5). However, rapid antigen detection methods have recently been introduced. Experiments with the IMAGEN™ direct immunofluorescence staining led to a positivity rate of 37.5% (6/16) for boar sperm samples and 27.5% (11/40) for sow vaginal swabs from intensive pig farms in Beijing (Tian *et al.*, 2012). Two studies used an in-house developed indirect ELISA to determine prevalence of chlamydial antigen in ruminant sera using a monoclonal antibody

(MAb) against a bovine *C. abortus* isolate. The first study reports a positivity rate of 25.8% (33/128) of tested goat sera and 32.2% (29/90) of tested calving cow sera in Ningxia (Xie *et al*., 2001). In the second study, all examined sheep (n=10) and cattle farms (n=13) in Ningxia tested positive. Average positivity rate was 22.1% (154/698) and 9.2% (107/1161) of examined sheep and cattle sera, respectively (Qiu and Xie, 2006). Unfortunately, we have no information on the species-specificity of the MAb used and neither of these two studies gave information on the health status of examined flocks.

As antigen detection methods often lack sensitivity and specificity, nucleic acid amplification assays were also developed recently by Chinese scientists. Several Chinese *Chlamydiaceae* isolates were characterised by *ompA* gene sequencing (Table 5). During an epizootic outbreak of cattle and caprine abortion in Taiwan, Wang *et al.* (2001) performed direct gene detection by *ompA*-based PCR using *C. abortus*-specific primers. Only 8.3% (1/12) of aborted calves and 33.3% (3/9) of aborted kids (young goats) tested positive by PCR, whilst seropositivity was as high as 71.4% and 58% in respective maternal sera samples (Tables 2 and 3). Moreover, PCR-positive samples were more often observed in vaginal swabs of healthy cows (45.2%; 14/31) and does (38.9%; 7/18) compared to cows (34.9%; 22/63) and does (21.4%; 8/24) that recently aborted. Yet, in contrast to successful isolation in 22.7% (5/22) and 33.3% (8/24) of PCR-positive vaginal swabs from affected cows and does, respectively, *Chlamydia* could not be isolated from any of the PCR-positive vaginal swabs of healthy animals. Two consistent point variations were found in all Taiwanese isolates, which shared 98.9 to 100% sequence identity, and bovine *C. abortus* strain LW508 (Wang *et al*., 2001). Recently, however, it became clear that *ompA* sequencing does not allow unambiguous identification of *C. psittaci* and *C. abortus*, which are phylogenetically highly related (Van Loock *et al*., 2003). Hence, it would be interesting to re-examine all characterised Chinese *C. abortus* and *C. psittaci* isolates with multilocus sequence typing and/or multilocus variable number of tandem repeat analysis in order to confirm the originally assigned species name.

Table 5. Isolation and molecular characterization by *ompA* sequencing of Chinese chlamydial isolates strains isolated from ruminants and swine

Strain	Year	Tissue	Species	Clinical symptoms	OmpA-sequencing	Reference
N	1981	gastric content, organs (aborted kid)	goat	abortion		(Shuai *et al*., 1981)
N	1983	liver (aborted lamb)	sheep	abortion		(Deng *et al*., 1983)
N	1984	aborted piglet	swine	abortion		(Yang *et al*., 1984)
N	1984	synovial fluid (piglet)	swine	polyarthritis		(Yang *et al*., 1984)
CW1	1986	aborted calf	cattle	abortion		(Yang *et al*., 1986)
CW2	1986	colostrum	cattle	abortion, mastitis	*C. psittaci* genotype C	(Yang *et al*., 1986; Song *et al*., 2009)
CW3	1986	milk	cattle	abortion, mastitis	*C. psittaci* genotype C	(Yang *et al*., 1986; Song *et al*., 2009)
N	1987	synovial fluid (lamb)	sheep	polyarthritis		(Xu *et al*., 1987)
N	1988	aborted calf (gastric content)	yak	abortion		(Shuai *et al*., 1988)
CCS-5 / CCS-10	1992	aborted calf	cattle	abortion		(Lin *et al*., 1992)
CYY1 / CYY2	1992	aborted calf	yak	abortion		(Lin *et al*., 1992)
NA	1997	aborted kid, placenta, vaginal swab	goat	abortion		(Liao *et al*., 1997)

Table 5-continued. Isolation and molecular characterization by *ompA* sequencing of Chinese chlamydial isolates strains isolated from ruminants and swine

Strain	Year	Tissue	Species	Clinical symptoms	OmpA-sequencing	Reference
HB1	1998	aborted piglet	swine	abortion	*C. psittaci*	(Qiu *et al*., 1998)
HB2	1998	vagina swab sow	swine	abortion	*C. psittaci*	(Qiu *et al*., 1998)
HB3	1998	piglet	swine	pneumonia, enteritis	*C. psittaci*	(Qiu *et al*., 1998)
CR99	2000	aborted piglet	Swine	abortion		(Li *et al*., 2000)
N	NA	NA	cattle/goat	abortion	*C. abortus* bovine strain LW508	(Wang *et al*., 2001)
LZ1	2006	milk	cattle	abortion	*C. psittaci*	(Qiu *et al*., 2006)
SX5	2006	liver (aborted calf)	cattle	abortion	*C. psittaci*	(Qiu *et al*., 2006)
NX	2006	gastric content (aborted calf)	cattle	abortion	*C. psittaci*	(Qiu *et al*., 2006)
N	2006	gastric content (aborted calf)	cattle	abortion		(Changqing *et al*., 2006)
CG1	NA	lung	sheep	pneumonia	*C. psittaci*	(Song *et al*., 2009)
CE1 / CE9	NA	NA	sheep	enteritis	*C. psittaci*	(Song *et al*., 2009)

N: No given name
NA: data not available

Similarly to scientists in other regions of the world, Chinese researchers also developed *Chlamydiaceae* specific 23S rRNA-based real-time PCR assays using SYBR Green fluorescence and the Lightcycler. The test developed by Yang *et al*. (2010) was highly sensitive as demonstrated by a detection limit as low as 250 fg of chlamydial DNA (*C. trachomatis*, *C. abortus*, *C. psittaci* and *C. pecorum*). Specificity was evaluated first by using a melting curve analysis followed by analysis of PCR products on agarose gels and nucleotide sequencing. Each of these analyses confirmed the specificity of the real-time PCR (Yang *et al*., 2010).

4. *Chlamydiaceae* clinical disease in Chinese livestock

Abortion is the most frequently observed clinical manifestation of chlamydiosis in Chinese ruminants. In Gansu, Tibet and Inner Mongolia, 20 to 25% of affected goat flocks aborted after approximately four months of gestation, which was often followed by placental retention in the aborting doe (Yang *et al*., 1981). Abortion rates in 9 Taiwanese farms with an outbreak of chlamydiosis ranged between 27 and 87% of pregnancies. Does did not show clinical signs prior to abortion, which generally occurred in the last two months of gestation (Liao *et al*., 1997). In sheep, interstitial pneumonia and polyarthritis in lambs was concomitantly observed with abortion and stillbirth in ewes (Shuai *et al*., 1988). In Hubei, dairy cattle aborted at 4 to 7 months of pregnancy and developed mastitis, whereas surviving calves showed symptoms of pneumonia, polyarthritis and conjunctivitis (Yang *et al*., 1986). In bovine abortion cases in Ningxia and Shaanxi, abortion typically occurred between 7 to 9 months of gestation and aborted fetuses were oedematous (Qiu *et al*., 2006). None of these reports mentioned presence of uterine discharge around the time of abortion, which commonly occurs in chlamydial abortion. Then again, period of abortion during gestation and lack of overt clinical signs prior to abortion is consistent with *C. abortus*. In Beijing, newborn calves developed polyarthritis, in which symptoms included fever, and tarsal and carpal swellings (Xiao *et al*., 2006). Symptoms observed in swine included polyarthritis in piglets (Yang *et al*., 1984), pneumonia in suckling piglets (Ren *et al*., 1994), and piglets dying from pneumonia and enteritis (Qiu, 2002). Furthermore, metritis, pneumonia, arthritis and diarrhoea were also observed in aborting sows (Yang *et al*., 1991). In horses, chlamydiosis was suspected to be present in mares producing

weak foals with pneumonia, joint swellings and diarrhea (Bao and Guo, 1992).

5. Prevention and treatment

5.1. Biosecurity

In China, farmers usually disinfect stables with a 2 to 3% sodium hydroxide dilution, 2 to 5% Lysol®, quaternary ammonium surfactants or a 0.5% sodium hypochlorite dilution. Implementation of these and other basic hygienic measures, such as the use of disinfecting foot baths and prevention of contact with susceptible animals alien to the group are feasible and applied in the closed environment of China's large-scale vertically integrated pig farms. For grazed or herded ruminants in extensive management conditions, these measures are, at best, limited to disinfection of equipment and prevention of contact between different herds or flocks. Similarly, acquisition of new stock from *C. abortus* free flocks or herds is also not feasible for small-scale farmers in rural Chinese areas where access to veterinary services are scarce.

5.2. Antibiotics and Chinese herbal medicine

Chlamydiaceae infections in livestock are in Western countries, principally treated with antibiotics. In China, however, herbal medicine is often used in conjunction with antibiotic treatment or applied as an alternative therapy. The latter is common in rural areas, where access to veterinary services and antibiotic treatment are scarce. These medicinal plant extracts are also used as prophylaxis. Chinese herbal medicine comes forth from century-old tradition and is often, but not exclusively, prescribed based on observed symptoms rather than as an etiological treatment.

Chlamydiaceae are sensitive to the broad-spectrum antibiotics tetracyclines, quinolones and macrolides (Mohamad and Rodolakis, 2010). In China, the antibiotics of choice for treatment of *Chlamydiaceae* infections in livestock are tetracyclines, followed by quinolone antibiotics. Additionally, the Chinese government has set maximum residue limits (MRLs) for tetracyclines and promulgated a government standard (GB/T 21317-2007) that established a method for its determination in animal tissues. The Chinese MRL of tetracycline in meat is 100 μg/kg (CNS, 2007). Moreover, preventive use of these antibiotics is highly discouraged to prevent

emergence of resistant, potentially zoonotic *Chlamydiaceae* strains. These regulations are similar to those in place in many other areas of the world.

In cattle and horses, Chinese herbal remedies for kerato-conjunctivitis caused by *Chlamydiaceae* include Xiao Huang San (clearing yellow swelling powder) and Jue Ming San (hallotis powder) (Table 6). Ingredients are ground into a fine powder or a decoction, which is followed by drying. Decoction refers to the mixture of filtered liquid of three consecutive cycles of boiling in water (Xie and Preast, 2010). Administration occurs twice daily by top-dressing on feed at a dose of 15 to 60 g Xiao Huang San during 1 month, or up to 50 g Jue Ming San during 1 to 2 months. Historically, all ingredients of Jue Ming San were ground into a fine powder and mixed with honey to form a paste for topical administration. Yu Benyuan and Yu Benheng, two noted veterinarians in the Ming Dynasty, first described the use of these herbal prescriptions in 1608 in Yuan Heng's Therapeutic Treatise of Horses. Xiao Huang San was originally developed for management of lameness in horses, but is currently regarded as the fundamental veterinary formula to treat painful swellings, including mastitis. Jue Ming San was developed to treat chronic ophthalmic inflammation. A 2009 Chinese report describes successful treatment of contagious kerato-conjunctivitis in cattle by use of either of these two herbal prescriptions (Luo and Abulaitepu, 2009). However, concurrent topical treatment with Ofloxacin, a second generation fluoroquinolone antibiotic, called into question the beneficial effects to be attributed to these herbal prescriptions.

Chinese herbal prevention and treatment of chlamydiosis in swine includes oral administration of a 1:1 mixture of plantain (*Plantago asiatica l.*, Cheqian) and white eclipta (*Eclipta alba*, Han Lian Cao) at a dose of 10 g per kg bodyweight during 14 days (Lin, 2002). In Chinese medicine, bactericidal and expectorant properties are ascribed to plantain (Ferris and Zheng, 1999). A major active compound in white eclipta is wedelolactone, for which an immunomodulating activity has been demonstrated. In Swiss albino mice, oral administration of methanol-extracted wedelolactone from white eclipta significantly increased (i) the phagocytotic index after intravenous injection of carbon ink, (ii) total white blood cell count after cyclophosphamide-induced myelosuppression, and (iii) humoral antibody titer after intra-peritoneal injection of sheep red blood cells (Jayathirtha and Mishra, 2004).

Table 6. Ingredient composition of Xiao Huang San (clearing yellow swelling powder) and Jue Ming San (haliotis powder).

Common name	Scientific name	Chinese name	Concentration (%)	
			Xiao Huang San	Jue Ming San
Glauber's salt (mirabilite)	$Na_2SO_4.10H_2O$	Mang Xiao	22.5	-
Abalone shell (halliotis shell)	*Halliotidis concha*	Shi Jue Ming	-	14.1
Chinese senna (sicklepod)	*Senna obtusifolia*	Jue Ming Zi	-	14.1
Chinese rhubarb	*Rheum palmatum*	Da Huang	7.5	9.4
Myrrh	*Commiphora myrrha*	Mo Yao	-	6.3
Goldthreat	*Coptis chinensis*	Huang Lian	7.5	6.3
Dioscera	*Dioscorea bulbifera tuber*	Hung Yao Zi	6.25	9.4
Stephania	*Stephania cepharantha hayata*	Bai Yao Zi	6.25	9.4
Weeping goldenbells	*Forsythia suspensae*	Lian Qiao	6.25	-
Anemarrhena	*Anemarrhena asphodeloicles*	Zhi Mu	6.25	-
Thunberg fritillaria bulb	*Fritillaria thunbergii*	Zhe Bei Mu	5	-
Membranous milk vetch	*Astragalus membranaceus*	Huang Qi	5	9.4
Gardenia jasmin	*Gardenia jasminoides*	Zhi Zi	5	9.4
Chinese skullcap	*Scutellaria baicalensis*	Huang Qin	5	6.3
Curcuma	*Curcuma spp.*	Yu Yin	5	6.3
Siler root	*Ledebouriella divaricata*	Fang Feng	5	-
Cicada moulting	*Cryptotympana atrata*	Chan Tui	3.75	-
Licorice root	*Glycyrrhiza uralensis*	Gan Cao	3.75	-

A matter of concern inherent to Chinese herbal medicine is that it largely relies on tradition

instead of scientifically sound efficacy and safety trials as required in Western medicine. Yet, although centuries of tradition are no proof of product efficacy and safety, it should neither be repudiated without prior investigation. It is indisputable that many herbs contain bioactive substances that can be associated with beneficial effects. Several of these secondary plant metabolites are synthetically produced for use in conventional medicine. However, herbal medicine lacks standardised dosing and involves a risk of co-administration of toxic plant compounds. Moreover, in contrast to conventional drugs, herbal medicines are not subjected to strict regulations and a mandatory quality control policy. The latter poses an additional safety risk that must not be underestimated. Heavy metal contamination, adulteration with undeclared synthetic drugs and cases of mistaken plant identity all have been repeatedly associated to severe adverse effects following use of Chinese herbal remedies (Rodolakis and Souriau, 1983).

6. Vaccination

In general, a strategy to exclude disease from chlamydial infections does not exist to date. Currently, commercial vaccines are only available for prevention of *C. abortus*. Moreover, all vaccines available in Europe and the USA are only licensed for use in sheep, immunisation in other ruminants being off-label usage. A present, the preferred strategy to control epizootic ovine abortion is through vaccination and keeping flocks closed, but implementation of these measures does not guarantee exclusion of infection and ensuing disease from the flock (Longbottom *et al.*, 2013a). These vaccines are not available in China, but the Lanzhou Veterinary Research Institute commercialises its own formalin-inactivated whole organism vaccine for use both in sheep and goat.

7. Public health significance of chlamydiosis in China

Zoonotic transmission of chlamydial species is described for *C. psittaci* from poultry and less frequently for *C. abortus* from small ruminants (Logbottom and Coulter, 2003). Globally, human acquisition of *C. psittaci* from mammals is yet to be substantiated. Similarly, zoonotic transfer of *C. pecorum* or *C. suis* has never been officially reported. Yet, preliminary results indicate that *C. suis* can be transmitted to humans (Vanrompay *et al.*, unpublished results).

To our knowledge, a confirmed case of zoonotic transfer of *C. abortus* has not been reported in China. Yet, Tian *et al*. (2012) described a possible transfer of *C. abortus* from swine to man in Beijing based on direct immunofluorescence staining (DIF) positivity of throat swabs from pig farmers (23.5%; 4/17) concomitant to DIF positivity of boar sperm (37.5%, 6/16) and sow vaginal swabs (27.5%, 11/40), as analysed by the IMAGEN^{TM} *Chlamydia* test. At the same time, *C. abortus* antibodies were also detected in serum of sampled swine using the ID Screen® *Chlamydia abortus* ELISA (Tian *et al*., 2012). However, *C. abortus* infections are yet to be demonstrated to be transmissible from swine to humans.

8. Conclusion

Virulent *Chlamydiaceae* strains are highly present in Chinese livestock, causing disease and abortion, and consequently, economic losses as in the rest of the world. Stringent surveillance is warranted in view of countervailing spread of Chinese isolates through export of live animals. A general lack of biosecurity measures implemented at extensive management systems in rural China is a matter of concern with respect to the control of chlamydial infections in livestock and the ensuing risk of zoonotic transfer. Another critical concern is the in China widely used practice of substitution of antibiotic therapy by herbal medicines that lack scientifically sound efficacy and safety trials, standardised dosing of active compounds, control on co-administration of toxic secondary plant metabolites, and a general mandatory quality control policy. Notwithstanding the inherent risks of inadequate treatment and even accidental intoxication following herbal treatment, it is indisputable that many herbs contain bioactive substances that can be associated with beneficial effects. Herbal remedies should therefore not be repudiated without prior investigation.

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Part B

Chlamydia psittaci infections in poultry: a review with emphasis on infections in China

Adapted from:
Lizi Yin[#], Isabelle Kalmar[#], Jeanne Boden and Daisy Vanrompay. Chlamydiosis in Chinese poultry. BMC Veterinary Research (submitted)
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Abstract

Chlamydia (*C.*) *psittaci* infections cause important economical losses to the poultry industry and are a danger to public health. The economic and zoonotic impact of chlamydial infections in the Western poultry industry is well documented. Less is known on its occurrence in Asia. Here we address chlamydiosis in Chinese poultry in view of China supplying 40.8% of global egg production and 14.2, 69.3 and 91.1% of global chicken, duck and goose meat, respectively. The current paper compiles English and Chinese literature on *Chlamydiaceae* infections in Chinese poultry. The paper is focusing on seroprevalence, culture, direct antigen detection, molecular characterization, observed symptoms, Chinese herbal medicine and psittacosis case reports. A review on the epidemiology of chlamydiosis in Chinese poultry clearly illustrates the widespread presence of virulent *Chlamydia* strains in chickens, ducks and geese across China. In Western countries, *Chlamydiaceae* infections in poultry are principally treated with antibiotics. In China, however, herbal medicine is often used in conjunction with antibiotic treatment or applied as an alternative hereto. The applied production and marketing systems facilitate zoonotic transfer. Chinese occupationally acquired psittacosis cases include reports on infections contracted from ducks, pigeons, chickens and peacocks. This confirms that the general belief of psittacosis being primarily associated with exposure to psittacine birds is a misconception.

Keywords: *Chlamydiaceae*, *Chlamydia psittaci*, poultry, China, psittacosis, zoonosis

1. Introduction

Chlamydiaceae are Gram-negative, obligate intracellular bacteria with a wide host range. Birds are the natural host of *Chlamydia* (*C.*) *psittaci*, which is extensively present in poultry, pet birds, feral pigeons and free-living birds. In addition, *C. psittaci* also occurs in mammals, for instance pigs and cattle (Eggeman *et al*., 2000; Vanrompay *et al*., 2004; Reinhold *et al*., 2011). Herein, a significant relation was found between *C. psittaci* infections in pigs and keeping poultry on pig farms (Eggeman *et al*., 2000; Vanrompay *et al*., 2004). The type strain for *C. psittaci* is 6BC (ATCC VR 125). In poultry, *Chlamydia* infections cause important economical losses and are a danger to public health. In fact, *C. psittaci* is the most important animal chlamydiosis with respect to zoonotic transfer. Transmission to humans occurs by inhalation or direct contact and may cause psittacosis or parrot fever.

China's poultry production and its share on the global market have increased significantly over the last decade. Epidemiological data, prophylactic and therapeutic measures taken as compared to Western practices, and zoonotic transfer of poultry diseases within China are thus of international importance. Chinese surveillance studies of chlamydiosis in poultry and case reports on zoonotic transfer are often published only locally in Chinese journals. Therefore, we consider both English and Chinese literature. As several Chinese reports lack an adequate description of applied diagnostic methods, only a selection of the most complete publications is provided here.

2. Molecular characterization of *C. psittaci*

2.1. *C. psittaci* ompA genotypes

C. psittaci infections have been found in all domestic bird species and at least 460 non-domestic bird species, representing 30 avian orders (Kaleta and Taday, 2003; Chahota *et al*., 2006). The 9 currently known outer membrane gene A (*ompA*) genotypes are designated into A to F, E/B, M56, and WC (Bush and Everett, 2001; Geens *et al*., 2005a). Genotypes A to F and E/B are primarily associated with chlamydiosis in birds; whereas M56 and WC are mammalian

pathogens. Yet, all genotypes should be considered to be readily transmissible to humans. Within the 7 avian genotypes, a predilection seems to be present for infection of specific bird orders. Genotype A and B strains, for instance, are endemic among psittacine birds (*Psittacidae*) and pigeons (*Columbiformes*), respectively. Waterfowl (*Anseriformes*) most frequently seem to be infected with genotype C strains, while genotype D strains are often associated with turkeys. Genotype E, also known as Cal-10, MP, or MN, was first isolated during an outbreak of pneumonia in humans during the early 1930s. Later on, genotype E isolates were obtained from a variety of bird species, including ducks, pigeons, ostriches, and rheas. Finally, genotype F is represented by the psittacine isolates VS225, Prk Daruma, 84/2334 and 10433-MA, but has also been isolated on a Belgian turkey farm (Van Loock *et al*., 2005). The mammalian M56 and WC genotypes were isolated during an outbreak in muskrats and hares, and during an outbreak of enteritis in cattle, respectively.

2.2. *C. psittaci* Multi-Locus Sequence Typing (MLST)

OmpA sequencing and even sequencing of the *rrn* spacer cannot always distinguish *C. psittaci* from *C. abortus* (Bush and Everett, 2001; Van Loock *et al*., 2003). Therefore, Pannekoek *et al*. (2010) used multi-locus sequence typing (MLST) for studying the population structure of *C. psittaci* and *C. abortus* (Pannekoek *et al*., 2010). The obtained MLST scheme was based on the partial sequences of seven housekeeping genes, *enoA*, *fumC*, *gatA*, *gidA*, *hemN*, *hflX* and *oppA*, representative for the whole genome. MLST of *C. psittaci* strains resulted in 11 unique sequence types (STs) (Table 1). MLST is extremely useful for distinguishing the phylogenetic highly related species *C. psittaci* and *C. abortus*, and is currently besides genome sequencing and high-resolution melting-curve (HRM) analysis the most reliable technique for species verification.

Table 1. MLST sequence types (STs) for different *C. psittaci ompA* genotypes.

Strain	Host	Country	*ompA* genotype	ST
6BC	Parakeet	California, USA	A	24
98/6098	Pigeon	Italy	B	26
CP3	Pigeon	California, USA	B	27
GD9	Duck	Germany	C	28
NJ1	Turkey	New Jersey, USA	D	37
92/1293	Turkey	The Netherlands	D	43
WS/RTE/3002/09/01	Duck	Germany	E/B	28
CPMN	Human	USA	E	35
VS225	Parakeet	Texas, USA	F	41
M56	Muskrat	Saskatchewan, Canada	M56	31
WC	Wolfsen cattle	California, USA	WC	32

3. The Chinese poultry industry

First, China's changing political orientation from 1985 onwards, facilitated its agricultural growth rate (Windhorst, 2008). Between 1985 and 2009, the annual poultry meat and egg production increased eightfold and fivefold in China; whereas only threefold and twofold on a global scale. This resulted in an increase of China's share on the global poultry meat and egg production from 6.5% to 17.8% and from 17.1% to 40.8%, respectively. Moreover, poultry meat production in China increased faster compared to pork production, through which its share increased from 9.6 to 21.1% on total meat production, the share of pork being lowered from 83.9% to 63.8%. The main poultry types reared for meat production in China include broilers, ducks and geese, representing 69.7% (11.4 MMT), 16.1% (2.6 MMT) and 14.26% (2.3 MMT) of total poultry meat production. The contribution of China to the global chicken, duck and goose meat production comprise 14.2%, 69.3% and 94.1%. In terms of gross production value calculated by multiplying gross production by output price at farm gate, chicken, duck and geese meat production in China account for 24,879; 6,439; and 5,663 million USD, respectively.

Next, although hen eggs encompass 85.1% (23.6 MMT) of total egg production, this accounts for only 54.8% (22,873 million USD) of total egg gross production value. Turkeys comprise a minor species with an annual production of only 2,946 Mt (FAOSTAT database, http://faostat.fao.org). Furthermore, China produces annually 700 million meat-type pigeons for consumption (Bu *et al*., 2010). *Per capita* meat consumption in China currently amounts 9.7 kg chicken meat, 4.1 kg beef and 37.3 kg pork, which is compared to *per capita* consumption in the US 21.8%, 10.9% and 139.2%, respectively (USDA database, http://www.fas.usda.gov). Likewise pork, egg consumption is traditionally high in China. Herein, *per capita* hen egg consumption in China compared to the global average was 21.3 kg (338 eggs) and 9.1 kg (144 eggs) in 2005, and is prospected to mount to 24.5 kg (389 eggs) and 9.8 kg (156 eggs) in 2015 (Windhorst, 2008). Because of China's high poultry production rate, even a low prevalence of productivity reducing infections results in considerable economic losses. Moreover, serious outbreaks may lead to a worldwide production-demand deficit.

Spatial distribution of poultry density within China (Figure 1), as well as the applied production and marketing systems, are essential factors to consider with respect to dissemination of infectious poultry diseases. Of great significance from an epidemiological point of view, clinical disease outbreaks of highly pathogenic avian influenza type H5N1 in China, for instance, have been associated with broiler and layer density. Whereas H5N1 presence identified by risk-based surveillance has been associated with density of domestic waterfowl, in which H5N1 is by far less pathogenic compared to chickens (Martin *et al*., 2011). To this respect, broiler and layer density are highest across eastern China, whereas ducks are primarily farmed in south-eastern China and Sichuan and geese in Sichuan and parts of Guangdong (Prosser *et al*., 2011). Furthermore, China encompasses three main poultry production systems, which greatly differ in size as well as level of biosecurity: traditional backyard farming, specialized householders and large-scale, vertically integrated enterprises (Bagust, 1994). In contrast to large-scale operations that use Western, high-productive breeds, the first two farming systems mainly rear indigenous breeds. The latter are on the one hand characterized by slow growth rate and poor laying performance, but on the other hand by higher resistance to harsh nutritional and environmental conditions (Hoffmann, 2005; Qu *et al*., 2006). In addition

to a better adaptation to free ranging systems in local geographic locations, Asian consumers prefer meat from traditional breeds, resulting in higher selling prices compared to meat from Western broiler strains (Hoffmann, 2005; Ding *et al*., 1999). Regardless of effects of the bird strain itself, differences in nutritional composition of diets and rearing period definitely contribute to the distinctive organoleptic traits of meat from traditional breeds. Slaughter at later age, for instance, results in increased total collagen and more collagen cross-linking, which in turn renders collagen less-soluble on heating, leading to loss of moisture and firmer texture (Wattanachant *et al*., 2004).

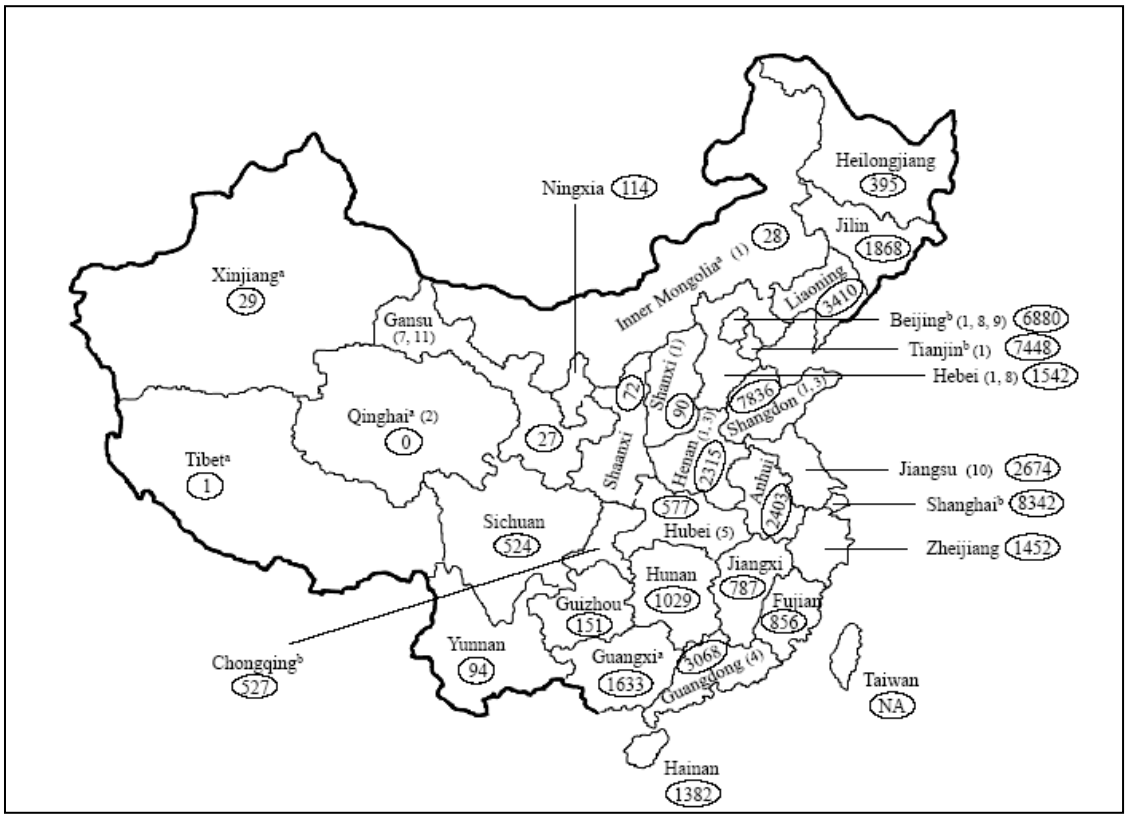

Figure 1. Poultry density (birds/km^2) in China (based on 2005 data in Bingsheng and Yijun, 2007)
[a] autonomous region; [b] municipality
available literature on seroprevalence of chlamydial antibodies is indicated: (1) Cao *et al*., 2006; (2) Jiang *et al*., 1993; (3) Li *et al*., 1992; (4) Lin *et al*., 2011; (5) Liu *et al*., 1990; (6) Shi *et al*., 2003; (7) Wang and Wei, 1991; (8) Yang *et al*., 2007; (9) Yang *et al*., 2011; (10) Yu *et al*., 1993; (11) Zhou *et al*., 2010

Practices inherent to these low-scale farming systems include birds scavenging of a backyard and herding of ducks onto harvested rice fields. This maximizes overlap between domestic poultry and wild birds, facilitating cross-species transmission of infectious diseases. As a result,

not only biosafety within these small-scale, extensive poultry operations is jeopardized, also an anthropogenic infectious threat is instigated towards wild birds, including endangered species and migratory birds (Lemus *et al*., 2010; Takekawa *et al*., 2010). On its turn, this poses an additional thread of spreading poultry-acquired diseases over considerable distances (Takekawa *et al*., 2010). Contact transmission of *C. psittaci* has been experimentally demonstrated in turkeys following group-housing with common grackles (*Quiscalus quiscula*) that were intra-tracheally inoculated with a turkey strain (Grimes, 1987). Even mammals may pose a wildlife reservoir for *C. psittaci.* This has been demonstrated by inoculation of turkeys with isolates obtained from the liver and spleen of an opossum and a domestic cat that scavenged on turkeys that died from avian chlamydiosis. The induced lesions in the experimentally infected turkeys were indistinguishable from those observed in turkeys suffering from avian chlamydiosis on site (Page, 1976).

4. Epidemiology of *C. psittaci* in Chinese poultry

The first case of chlamydiosis in Chinese poultry dates back to 1959. Few outbreaks were reported up to the 1980s. Increased outbreaks in Chinese poultry during the 1980s and 1990s coincided with outbreaks in U.S. turkeys during the 1980s and in European turkeys during the 1990s. This could in part be attributed to higher awareness of the economical and zoonotical impact of chlamydiosis on the fast growing Chinese poultry industry, resulting in increased surveillance. But in general, it was believed that these observed increased incidence rates were real (Zhou and Qiu, 2007).

4.1. Seroprevalence

So far, sero-surveillance of avian chlamydiosis by Chinese investigators occurred primarily by indirect haemagglutination assays (IHA). In 2011, for instance, IHA was still used in avian livestock sampled in Guangdong. Seroprevalence was 6% (12/ 200) in domestic ducks obtained from a live market, 10.8% (13/120) in free-range, 6 month old chickens obtained from two slaughterhouses, 25% (30/120) in caged 45 to 60 day old chickens from four commercial flocks, 21.8% (31/142) in farm-bred geese, 22.4% (13/58) in house-bred geese, and 17% (34/200) in

meat-type pigeons obtained from seven commercial flocks (Lin *et al*., 2011). Prevalence data of chlamydial antibodies obtained by IHA in chickens, ducks and geese are available for, respectively, 13, 10 and 2 of China's 32 provinces, autonomous regions and municipals (Table 2).

Table 2. Seroprevalence (%) of chlamydial antibodies in Chinese poultry.

Year	Species	Province	Methods	Seroprevalence	Reference
1990	chicken	Hubei	IHA	25.6 (287/1121)	Liu *et al*., 1990
1990	duck	Hubei	IHA	10.2 (97/950)	Liu *et al*., 1990
1990	goose	Hubei	IHA	8.9 (53/594)	Liu *et al*., 1990
1991	chicken	Gansu	IHA	26.9 (173/643)	Wang and Wei, 1991
1992	chicken	Shandong	ICF	11.9 (56/470)	Li *et al*., 1992
1992	chicken	Henan	ICF	18.3 (15/82)	Li *et al*., 1992
1993	chicken	Qinghai	IHA	0.6 (2/362)	Jiang *et al*., 1993
1993	chicken[a]	Jiangsu	IHA	63.7 (429/673)	Yu *et al*., 1993
2003	broiler[a]	Beijing	IHA	30 (15/50)	Shi *et al*., 2003
2006	SPF chicken	7 provinces[b]	IHA	0 (0/14)	Cao *et al*., 2006
2006	SPF chicken	7 provinces[b]	ELISA	100 (10/10)	Cao *et al*., 2006
2006	chicken	7 provinces[b]	IHA	26.3 (49/186)	Cao *et al*., 2006
2006	chicken	7 provinces[b]	ELISA	72.5 (58/80)	Cao *et al*., 2006
2006	duck	7 provinces[b]	IHA	25.3 (44/174)	Cao *et al*., 2006
2006	duck	7 provinces[b]	ELISA	82.0 (41/50)	Cao *et al*., 2006
2007	duck	Hebei	IHA	50.0 (52/104)	Yang *et al*., 2007
2007	duck	Hebei	ELISA	40.0 (12/30)	Yang *et al*., 2007
2010	chicken[a]	Gansu	IHA	26.9 (7/26)	Zhou *et al*., 2010
2011	peacock[a]	Beijing	IF	60.0 (12/20)	Yang *et al*., 2011
2011	duck	Guangdong	IHA	6.0 (12/200)	Lin *et al*., 2011
2011	chicken	Guangdong	IHA	17.9 (43/240)	Lin *et al*., 2011
2011	goose	Guangdong	IHA	22.0 (44/200)	Lin *et al*., 2011
2011	pigeon	Guangdong	IHA	17.0 (34/200)	Lin *et al*., 2011

[a]flocks with clinical symptoms consistent with *Chlamydia psittaci*

[b]Beijing, Hebei, Tianjin, Inner Mongolia, Shandong, Shanxi and Henan

SPF: specific-pathogen-free; IHA: indirect haemagglutination assay; ICF: indirect complement fixation test; ELISA: enzyme-linked immunosorbent assay; IF immunofluorescence test

First use of an enzyme-linked immune sorbent assay (ELISA) for detecting *C. psittaci* antibodies in Chinese poultry was in 2006. Results of the RIDASCREEN *C. psittaci* antibody ELISA (R-Biopharm, Darmstadt, Germany) were compared with those obtained by an in China widely used commercial IHA kit (Lanzhou Veterinary Research Institute, China). Seroprevalence data obtained by ELISA were much higher in all tested poultry species: 100% versus 0% in specific-pathogen-free (SPF) chickens, 78% versus 30.8% in laying hens, 66.3% versus 20.2% in meat-type chickens, and 82% versus 25.3% in ducks (Cao *et al*., 2006). In Hebei ducks, the discrepancy between results obtained by RIDASCREEN ELISA and IHA was less pronounced, revealing seroprevalences of 50% and 40%, respectively (Yang *et al*., 2007). Only one study makes reference to use of a semi quantitative immunofluorescence (IF) assay, in which 60 % (12/20) of peacocks with respiratory distress showed to be positive (Yang *et al*., 2011).

4.2. *C. psittaci* culture and direct antigen detection

The obligate intracellular nature of *Chlamydiaceae* necessitates transportation in appropriate medium to maintain viability, and culture in yolk sac of SPF chicken embryos or in cell lines. In addition, culture requires a biosafety level three facility, which limits the number of laboratories authorized for diagnosis by isolation (Beeckman and Vanrompay, 2010). Only few reports mentioned diagnosis of avian chlamydiosis by culture or direct antigen detection in China.

Shi *et al*. (2003) described symptoms of anorexia, rhinitis, conjunctivitis and respiratory distress in broiler farms in Beijing and Tianjin. Together with *E. coli*, *C. psittaci* was regarded as the etiological agent. They isolated *C. psittaci* from lung tissue using yolk sac inoculation of 7-day-old SPF chicken embryos. Interestingly, some years later, Van Loock *et al.* (2006), proved the existence of a pathogenic interplay between *C. psittaci* and *E. coli* in experimentally infected SPF turkeys. Chlamydial antigen was detected in 12 of 20 pharyngeal swabs and 4 of 5 lung samples of peacocks suffering from respiratory distress using a direct IF test. Culture of positive samples in McCoy cells resulted in a typical cytopathic effect 48 hours post infection, and chlamydial inclusions were observed in one lung sample after a second passage (Yang *et al*., 2011). In ducks and chickens with tubal cysts, low egg production and pneumonia, modified

Ziehl-Neelsen staining showed a positive result in 18.5% of tested samples, whereas an IF test detecting chlamydial lipopolysaccharide (LPS) (IMAGEN, *Chlamydia*, Dako Cytomation, UK) revealed a positive rate of 38.2% (Cao *et al*., 2006). This finding is in accordance to earlier data in which a higher sensitivity and specificity is demonstrated in IF assays compared to cytological staining (Vanrompay *et al*., 1992).

4.3. Molecular characterization of Chinese *C. psittaci* isolates

Four reports published in international journals mentioned molecular characterization of chlamydial isolates in Chinese poultry, all by sequencing the *ompA* gene encoding the major outer membrane protein (MOMP). Zhang *et al*. (2008) characterized isolates obtained from oviduct cysts of laying hens with poor egg production as genotype C. Genotyping by Song *et al*. (2009) of a not further specified duck isolate that was obtained from the Beijing Veterinary Diagnosis Institute also revealed genotype C. The *ompA* encoded MOMP of the isolate obtained from the liver and spleen from laying hens showing reduced laying performances in Lanzhou city (Gansu) revealed highest amino acid homology (94.4%) with the MOMP of *C. psittaci* strainVS225 (Zhou *et al*., 2010). The latter is a genotype F strain that was first isolated from a parakeet in Texas (US) and is still mostly isolated from parrots (Andersen, 1997; Van Loock *et al*., 2003). To the authors' knowledge, these two reports are currently the only published records on the occurrence of respectively genotype C and genotype F in chickens. However, genotype F has previously been isolated from turkeys (BUT/T9/Webster) originating from France and raised in Belgium (Van Loock *et al*., 2005). The latter isolate has been associated with zoonotic transmission (Van Droogenbroek *et al*., 2009). Yang *et al*. (2011) sequenced the *ompA* gene of isolates obtained from peacocks (pharyngeal swabs and lung tissue) and peacock farmers (throat swabs) suffering from respiratory distress. Although the *ompA* gene sequence from the isolates showed high similarity with *C. psittaci* strains Cal-10 (99.4% homology, genotype E) and 6BC (99.2% homology, genotype A), the authors concluded genotype B based on the outdated restriction fragment length polymorphism (RFLP) method. High nucleic acid homology of the *ompA* gene of this isolate with both genotype E and A strains demonstrates the limitations of *ompA* sequencing in molecular characterization of certain *Chlamydia* isolates, for which multi-locus sequence typing is thus advisable.

Two additional studies, published in Chinese, report molecular characterization of the *ompA* gene of Chinese *C. psittaci* isolates. First, a not further specified Inner Mongolian isolate was reported to show 91.9% and 83.1% homology of *ompA* with *C. psittaci* strains TT3 (genotype D) and 6BC (genotype A), respectively (Wang *et al*., 2007). Second, sequencing of the *ompA* gene of isolates obtained from pharyngeal swabs, air sacs and uterine mucosae of commercial and SPF laying hens, broilers and ducks revealed over 99.0% homology with *C. psittaci* strain 6BC (genotype A) (Zhang *et al*., 2008).

5. Clinical signs and macroscopic lesions

Outbreaks of avian chlamydiosis in meat-type chicken farms reported in Beijing and Tianjin showed a morbidity of 40% in 2001 and 30% in 2002. Observed clinical symptoms included anorexia, head shaking (rhinitis), a watery discharge from the eyes (conjunctivitis), dyspnoea and coughing. Autopsy findings were pneumonia, opacity of the air sacs and severe pericarditis (Shi *et al*., 2003). Yu *et al*. (1994), and more recently Zhou *et al*. (2010), described chlamydiosis in Chinese layer-type chickens. The morbidity on diseased farms ranged from 20 to 30%. Clinical presentation was a drop in egg production up to 50%, malformed eggs, anorexia, weight loss, depression, diarrhoea and ascites. Autopsy revealed oviducts filled with colourless inflammatory liquid, pneumonia, air sac opacity, pericarditis and hepatosplenomegaly (Zhou *et al*., 2010). In breeder-type ducks, chlamydiosis was observed to initially present as increased tear production, wet feathers around the eyes and conjunctival flushing, followed by diarrhoea characterized by watery, light green droppings, and marked weight loss. Autopsy of sick ducks revealed perihepatitis and hepatosplenomegaly (Yang *et al*., 2007). Meat-type ducks of age 120 days infected with *C. psittaci* in a farm in Beijing showed depression, loss of appetite, weight loss, ataxia, tremor, yellow-green droppings, serous or purulent discharge around the eyes and noses, difficult breathing and dead. Autopsy findings were conjunctivitis, keratitis, ocular atrophy, rhinitis, pericardial effusion, liver necrosis, cloudy air sacs, hepatosplenomegaly, pulmonary congestion and intestinal bleedings (Xiao *et al*., 2008). In peacocks, clinical presentation included anorexia, weight loss, yellowish droppings, conjunctivitis, sinusitis, pneumonia and mortality. In cases with a fatal course of infection,

mortality usually occurred within 7 days after first appearance of symptoms (Yang *et al*., 2011). All together, these data clearly show that *C. psittaci* infections in economically important avian species of China's poultry industry induce symptoms severe enough to warrant awareness, surveillance, prophylaxis and proper treatment.

6. Prevention and treatment of avian chlamydiosis in China

6.1. Prophylaxis

In contrast to *C. felis* and *C. abortus* for which live attenuated and inactivated whole organism vaccines are commercially available, there are no registered vaccines against *C. psittaci* to date (Longbottom and Livingstone, 2006; Masubuchi *et al*., 2010). Yet, literature data on experimental vaccines against avian chlamydiosis show promising results. Interestingly, in contrast to the commercially available vaccines against other chlamydial species, the majority of reported experimental vaccines against avian chlamydiosis are subunit vaccines based on the immuno-dominant MOMP or its encoding DNA (Verminnen and Vanrompay, 2009). Lack of availability of prophylactic vaccines and the general advice against routine preventive use of antibiotics highlights the importance of implementing biosafety measures to avoid introduction of *C. psittaci* into the flock and to preclude continued dissemination between production rounds when infection occurs.

6.2. Antibiotic treatment

Chlortetracycline (CTC) and doxycycline are worldwide most often used against avian chlamydiosis. In China, treatment of chlamydiosis in poultry generally consists of a short 3 to 5 day treatment period with tetracycline hydrochloride at a dose of 25-50 mg/kg (VPC-PRC, 2000). The Chinese government has set maximum residue limits (MRLs) for tetracyclines in muscle (100μg/kg) and promulgated a government standard (GB/T 21317-2007) that established a method for the determination of tetracyclines in animal tissues (Chinese National Standard, 2007).

6.3. Chinese herbal medicine

Besides therapeutic use of antibiotics, China also harbours the practice of formulations based on extracts of medicinal plants. The herbal prescriptions Maxing Shigan San and Chuanyichai San are used as prophylactic and therapeutic treatment against avian chlamydiosis (Xiao *et al*., 2008; Wang, 2001). Critical concerns inherent to Chinese herbal medicine include: i) lack of scientifically sound efficacy and safety trials, ii) lack of standardized dosing of active compounds, iii) co-administration of toxic secondary plant metabolites, and iv) lack of a mandatory quality control policy. It is, however, indisputable that many herbs contain bioactive substances that can be associated with beneficial effects. Herbal remedies should therefore not be repudiated without prior investigation.

For treatment of chlamydiosis in poultry, Maxing Shigan San is added to the feed or drinking water for 3 to 5 days at a dose of 1 to 1.25 g/kg and 1 g/L, respectively. Halve these doses are used for prophylaxis. The ingredient composition is ephedra (*Ephedra sinica*, Ma Huang), bitter almond (*Prunus armeniaca*, Ku Xing Ren), plaster stone (*Gypsum fibrosum*, Shi Gao) and licorice root (*Glycyrrhiza Uralensis*, Gan Cao) in a proportion of 1:1:5:1 (VPC-PRC, 2000). Since thousands of years, the desert dwelling shrub ephedra has been widely used in Chinese herbal medicine to tread common colds, asthma, hay fever, and hypotension (Pettis *et al*., 2004). The main active constituents include at least six phenylpropylamino alkaloids, also known as ephedrine alkaloids: (1R, 2S)-norephedrine, (1S, 2S)-norpseudoephedrine, (1R, 2S)-ephedrine, (1S, 2S)-pseudoephedrine, (1R, 2S)-N-methylephedrine and (1S, 2S)-N-methylpseudo-ephedrine (Krizevski *et al*., 2010). Ephedrine, similarly to epinephrine, stimulates the α and β adrenergic receptors, resulting in bronchodilatation and in an increase in systolic and diastolic blood pressure. Furthermore, the chemical structure of ephedrine alkaloids shows high similarity to the synthetic stimulant methamphetamine. In contrast to epinephrine, but likewise methamphetamine, ephedrine also stimulates the central nervous system (Pettis *et al*., 2004). Finally, essential oils in ephedra are attributed to possess anti-pyretic effects. In Maxing Shigan San, bitter almond is added to reduce dyspnoea in synergy with ephedra. The anti-pyretic properties of ephedra are increased by addition of plasterstone or *Gypsum fibrosum* (Chen and

Chen, 2004). The main constituent of plasterstone is calcium sulfate ($CaSO_4.2H_2O$), which is in Chinese medicine considered to be anti-allergenic, to support macrophage function and strengthen the immune response, and to have weak anti-pyretic properties. In general, it is mainly used in combination with synergistic herbs to increase its anti-pyretic properties (Huang and Michael, 2009). Licorice is found in Central Asia, Mongolia and China. In China, it is considered 'the king of herbs', and is one of the most used herb species in herbal medicine. The dried root of licorice contains glycyrrhizin, a triterpenoid saponin glycoside to which several favourable properties are attributed, including expectorant and anti-pyretic activities, detoxification, anti-oxidative, antibacterial and antiviral effects (Fiore *et al*., 2008; Hayashi and Sudo, 2009; Huo *et al*., 2011). It is also worldwide used in the candy industry, being present in, for instance, Super Ropes®, and Natural Vines® candies (American Licorice company, Union City, CA), Mentos® Drop Citroen and Mentos® Drop Aardbei. Actually, drop is the generic name for licorice in Dutch.

The second Chinese herbal medicine used against avian chlamydiosis is Chuanyichai San. Its ingredient composition is in decreasing order: barley (*Hordeum vulgare*), dyer's wood leaf (*Isatis indigotica*), honeysuckle flower (*Lonicerae flos*), Chinese angelica (*Angelica sinensis*), fritillaria bulb (*Fritillariae cirrhosae*), Chinese parsley root (*Ligusticum wallichii*), thorowax root (*Bupleurum chinense*), goldthread (*Coptis chinensis*), licorice (*Glycyrrhiza Uralensis*) and ephedra (*Ephedra sinica*) (Table 3). Dyers' wood leave contains biologically active indoxyl derivatives with anti-inflammatory and anti-viral properties (Zou and Koh, 2007). Flowers of honeysuckle contain chlorogenic acid (3-caffeoyl-D-quinic acid), to which anti-oxidant, anti-microbial, anti-mutagenic and anti-inflammatory properties have been attributed. In Chinese herbal medicine, besides treatment of epidemic febrile diseases, honeysuckle flower is also used for treatment of sores, carbuncles and furuncles. In comparison, plant-derived chlorogenic acid is also used in the cosmetic industry (Xiang and Ning, 2008). The subsequent ingredient is dried root of the indigenous herb Chinese angelica, which has a long history in herbal Chinese medicine as a remedy for women's disorders. It has been reported that extracts exert anti-bacterial and anti-inflammatory effects as well as anti-proliferative effects on tumour cells, reduction of oxidative stress, and protection of cardiomyocytes against oxidant injury by

increasing cellular glutathione (Han and Guo, 2012). The processed bulb of *Fritillaria*, which is also used in Chinese cough remedies, contains alkaloids and modulates airway inflammation by suppression of cytokine, immunoglobin E and histamine production, and eosinophilic accumulation along with increased interferon-gamma production in tests on lung tissue (Yeum *et al*., 2007). Next, Chinese parsley root is a Chinese flowering plant of the carrot family containing the phyto-chemicals: ligustilide, cnidilide, neo-cnidilide and sedanolide. Attributed activities include: cholesterol reduction, anti-arteriosclerotic, anti-spasmodic, anti-bacterial, anti-fungal, cardiotonic, vasodilative, hypotensive, tranquilizing, anti-coagulant, estrogenic uterine stimulant and emmenagogue (Zheng *et al*., 2011). Thorowax root, a medicinal root found natively in East Asia, is added for its ascribed detoxifying and anti-microbial properties. Its use was already recorded in the Imperial Grace Formulary of the Tai Ping Era (Tai Ping Hui Min He Ji Ju Fang), Imperial Medical Department, 1078-1085 AD. The active ingredients include saponins and plant sterols, which have been shown to lower fever and reduce inflammation in animal studies. The medicinal use of *Coptis chinensis*, a species of goldthread native to China, was first recorded in Divine Husbandman's Classic of Materia Medica (Shen Nong Ben Cao Jing). The root of this plant is a source of isoquinoline alkaloids like berberine, palmatine, hydrastine, and coptisine. In herbal Chinese medicine, it is used for removing evil heat, draining dampness, draining fire and relieving toxicity (Chen and Chen, 2004). More recent pharmacological studies indicate that goldthread has anti-bacterial and anti-inflammatory effects and may improve immune functions (Kamath *et al*., 2009). Common herbs in both traditional formulas to treat avian chlamydiosis are licorice and ephedra.

Table 3. Ingredient composition of Chuanyichai San

Common name	Scientific name	Chinese name	Concentration (%)
Barley	*Hordeum vulgare*	Mai Ya	50
Dyer's woad lea	*Isatis indigotica*	Da Qing Ye	25
Honeysuckle flower	*Lonicerae flos*	Jin Yin Hua	5
Chinese angelica	*Angelica sinensis*	Dang Gui	5
Fritillaria bulb	*Fritillariae cirrhosae*	Chuan Bei Mu	3.5
Chinese parsley root	*Ligusticum wallichii*	Chuan Xiong	2.5
Thorowax root	*Bupleurum chinense*	Chai Hu	2.5
Goldthread	*Coptis chinensis*	Huang Lian	2.5
Licorice	*Glycyrrhiza Uralensis*	Gan Cao	2.5
Ephedra	*Ephedra sinica*	Ma Huang	1.5

7. Psittacosis in China

Over the years, psittacosis or *C. psittaci* infections in humans has been reported worldwide. Since 2008, psittacosis is enlisted as a notifiable infectious disease in Hong Kong, and since then, 4 confirmed cases and 3 additional probable cases have been reported (http://www.chp.gov.hk). Furthermore, 2 cases were confirmed by the Taiwan Centre for Disease Control. Official statistics on prevalence of psittacosis on the mainland of China, however, are not readily available. As psittacosis is generally considered to be extremely rare and because a sensitive and specific diagnosis of an on-going infection with this obligate intracellular organism requires highly specialized techniques, adequate diagnostic tests are not routinely performed. Hence, reported cases probably represent only the tip of the iceberg. Severe infections with *C. psittaci*, involving pneumonia with or without cardiac and/or neurological complications, may be correctly diagnosed as psittacosis. However, less severe cases, characterized by flu-like symptoms and asymptomatic infections, are prone to remain

largely underdiagnosed and underreported. Moreover, reported cases worldwide are generally highest in the older age groups, which may reflect increased investigation and laboratory testing for atypical community acquired pneumonia in this age group. Also, the common misconception that psittacosis is associated only with exposure to psittacine birds might lead to negligence of laboratory testing for psittacosis in clinically compatible cases without history of contact with parrots (Yohannes *et al*., 2006).

In China, likewise elsewhere, a confirmed case of psittacosis refers to a clinical compatible case that is laboratory confirmed by isolation of the causative agent or demonstration of its DNA in respiratory secretions, or by at least a fourfold increase in antibodies against *C. psittaci* between paired acute and convalescent sera collected at least 2 weeks apart. Interestingly, prevalence data from the Hong Kong Special Administrative Region and the Province of Taiwan are extreme low compared to, for instance, the number of cases reported by the US Centers for Disease Control, which amounts 2,393 confirmed cases between 1978 and 2011 (http://wonder.cdc.gov). Off course, official US data dates back from much earlier, when up to 1990, over 100 cases were annually reported, whilst cumulative data for 2008 to 2011 amounted only 24 cases. The lower number of cases in recent years might reflect enhanced control measures and use of improved diagnostic tests that better distinguish *C. psittaci* from more common *C. pneumonia* infections (CDC, 2009). Still, one could expect a much higher number of actual cases in China compared to Western countries. In the latter, close contact with food-producing animals and thus an increased risk for psittacosis is mainly confined to employees in poultry farms or poultry processing plants, and veterinarians. In contrast, in rural areas of China, most people live in close contact with live birds due to the widespread backyard systems, which moreover typically harbour a low level of biosecurity. In addition, high-risk behaviour of consumers on live markets in urban areas, such as, blowing the cloacae of chickens to examine their healthiness and preference for slaughtering on site or at home, further expands the zoonotic risk of *C. psittaci* to a wider public, instead of being mainly confined to an occupational hazard (Woo *et al*., 2006).

Up to date, reported cases outside China are mostly related to contact with psittacine birds or

turkeys and ducks (Harkinezhad *et al*., 2009; Beeckman and Vanrompay, 2010b). Cases confirmed by the Taiwan Center for Disease Control were suspected to be related to contact with psittacine pet birds (http://www.cdc.gov.tw). Literature data on occupationally acquired psittacosis in China includes reports on infections contracted from meat-type ducks, meat-type pigeons, chickens and peacocks.

Most cases are published locally in Chinese journals. As several of these reports lack an adequate description of applied diagnostic tests, only a selection of the most complete publications is provided here. The earliest report dates from 1959, in which psittacosis was diagnosed in 7 of 13 employees of a meat-type duck farm in Beijing (Zhou and Qiu, 2007). Affected employees showed flu-like symptoms and pneumonia. Serum was examined by CFT and all 7 cases had an antibody titre of 1:32. This is of course no proof of an on-going chlamydia infection. The examination of convalescent sera would have been more appropriate. Also related to contact with ducks, Zhang and Yang (2009) reported two cases of psittacosis after manipulating dead ducks on a farm in Sichuan. Both employees developed fever, headache, chest pain, dyspnoea and diarrhoea, and were treated with azithromycin (Zhang and Yang, 2009). Although this report was published in 2009, psittacosis was diagnosed by clinical symptoms, history of contact with diseased ducks and exclusion of avian flu and SARS, which are by far exclusive diagnostic tools. Occupationally contracted psittacosis was also reported after contact with meat-type pigeons in Beijing (Wang *et al*., 2007; Gao *et al*., 1989). In 1987, three employees of a pigeon farm in Beijing developed fever, a sore throat and a dry cough. They were diagnosed as psittacosis by positive CFT and recovered after treatment with erythromycin. Differential diagnosis with rickettsial infections were made by the Weil-Felix test (Pettis *et al*., 2004). In another Beijing pigeon farm, 5 of 20 employees developed fever, cough, a sore throat and chest pain after contact with newly acquired pigeons and were diagnosed as psittacosis by CFT using paired sera (Gao *et al*., 1989). Another publication in Chinese reported psittacosis diagnosed by CFT on paired sera in an engineer employed on a chicken farm (Sun, 2010).

Finally, a report on psittacosis in employees of a Chinese peacock farm was published in an international journal. Four employees developed high fever and respiratory distress during an

outbreak of pneumonia, airsacculitis, diarrhoea and mortality in peacocks. Throat swabs were taken during the course of their illness and blood samples were collected 20 days later. Chlamydial antigen was detected in 1 of 4 throat swabs by a direct immunofluorescence test. Sera of all four employees were positive for immunoglobin G antibodies against *C. psittaci* in 1:64 diluted samples using a semi-quantitative immunofluorescence test (Yang *et al*., 2011).

Reported prevalence data of *C. psittaci* antibodies in IHA-tested sera of healthy human populations in China were 200 of 2915 (6.86%) tested random volunteers in Xinjiang (Jin *et al*., 1993), 11 of 298 (3.69%) of tested poultry workers in Shandong (Yang, 1993), and 44 of 676 (6.51%) tested slayers, feeders, veterinarians and milk processors in Yunnan (Dou *et al*., 1995). In another study, presence of immunoglobulin G against *C. psittaci* at a level ≥ 1:16 using a micro-immunofluorescence (MIF) test revealed a positive result in 25 of 711 (3.5%) healthy volunteers and 3 of 51 (5.9%) healthy employees of a poultry-raising and processing plant in Fujian. However, using the same test in Beijing, prevalence of antibodies against *C. psittaci* was much higher in female prostitutes (15 of 106, 14.2%), attendants of an STD clinic (10 of 98, 10.2%) and patients suffering from pneumonia and bronchitis (8 of 108, 7.4%). Moreover, the results indicated that prevalence of antibodies against *C. trachomatis* (13.8%) and *C. pneumonia* (62.7%) was much higher in poultry workers than prevalence of antibodies against *C. psittaci* (5.9%). Also, in attendants of an STD clinic, prevalence of antibodies against *C. pneumonia* was about threefold (66%) compared to *C. trachomatis* (23.5%) (Ni *et al*., 1996). These figures strongly suggest cross-reactivity and indicate inadequate specificity of the MIF test, which uses formalin-fixed whole elementary bodies and primarily detects antibodies directed against surface antigens, such as genus-specific LPS epitopes.

8. Conclusion

Inherent to its vast extend, China's poultry sector is even at low prevalence highly vulnerable to massive economic losses due to disease provoking infectious agents. In addition, the likelihood of infectious outbreaks is increased by the vast amount of small-scaled backyard production systems that hardly implement any biosafety measures. High-risk behavior of consumers and the nature of China's applied agricultural and marketing systems further facilitate cross-species

transmission, increasing the public health risk as well as the ecological hazard to endangered wildlife. To this respect, *C. psittaci* is one such pathogen that encompasses economic, zoonotic and ecological hazards.

Diagnosis of avian chlamydiosis and psittacosis in humans is impeded due to the obligate intracellular nature of the bacterium, which necessitates isolation on cell culture or on yolk sac of embryonating eggs. Another diagnostic difficulty is cross-reactivity of most currently available serological tests with family members or other Gram-negative bacteria. Moreover, symptoms are not pathognomonic. Although the acquisition of seroprevalence data on *C. psittaci* in China often still occurs through outdated methods, it is clear that *C. psittaci* is widespread in China's poultry industry. Furthermore, economically important implications of the occurrence of virulent strains have been repeatedly demonstrated throughout China. Vaccines against *C. psittaci* are not commercially available to date. In China, avian chlamydiosis is either treated with tetracycline antibiotics, likewise elsewhere, or by Chinese herbal medicines. Published results on efficacy or safety of the latter, however, are lacking.

Reported cases of psittacosis, which is also in China a notifiable disease, probably represent only the tip of the iceberg because diagnosis requires highly specialised techniques that are not routinely performed. Still, Chinese occupationally acquired psittacosis cases include reports on infections contracted from ducks, pigeons, chickens and peacocks. This confirms that the general belief that psittacosis is primarily associated with exposure to psittacine birds is a misconception. Negligence of laboratory testing for psittacosis in clinically compatible cases without history of contact with parrots is thus outdated.

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Chapter Two

Prevalence of *Chlamydia abortus* in Belgian ruminants

Running title**:** *Chlamydia abortus* in Belgian ruminants

Adapted from:

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Abstract

Chlamydia (*C.*) *abortus* induced lamb loss and enzootic abortion during the last third week of gestation remains the most common cause of reproductive failure in sheep-breeding countries all over the world. *C. abortus* in cattle is predominantly associated with genital tract disease and mastitis. We examined the occurrence of *C. abortus* antibodies in Belgian sheep (n=988), goat (n=48) and cattle (n=1887) using the ID Screen™ *Chlamydia abortus* indirect multi-species ELISA. Sampling herds at n ≥ 10 revealed a seropositive herd status in 14.3% (6/42), 50% (1/2) and 11.6% (11/95) of sheep, goat and cattle herds, respectively. Seroprevalence in one goat herd was 52.9% (9/17), but prevalence in positive sheep and cattle herds was generally limited to only 1 or 2 seropositive animals on 10 to 20 tested animals per herd. Molecular diagnosis by the 23S RNA-based ArrayTube™ microarray on rectal swabs sampled at 3 cattle farms (n=20 per farm) tested all negative for *Chlamydiaceae* DNA.

Keywords*: Chlamydia abortus*, sheep, cattle, goat, ruminants, Belgium.

1. Introduction

Chlamydiaceae are Gram-negative, obligate intracellular bacteria. Recently, the assignment from the single genus *Chlamydia* into two genera, *Chlamydia* and *Chlamydophila* by Everett *et al*. (1999) has been reconsidered. Based on comparative genome analysis of several *Chlamydiaceae* genomes, the *Chlamydiaceae* are currently back reunited into a single genus, *Chlamydia* (Kuo and Stephens, 2011).

Ruminants can become infected with *Chlamydia* (*C.*) *abortus*, *C. pecorum*, *C. psittaci* and although rarely *C. suis* (reviewed by Reinhold *et al*., 2011). *Chlamydiaceae* infections in cattle (*Bostaurus*) have been known to occur since 1940, when McNutt isolated intracellular organisms from cases of sporadic bovine encephalomyelitis in feedlot cattle (McNutt and Waller, 1940). Thereafter, a number of studies worldwide reported epizootic bovine abortion in cattle caused by *C. abortus* (reviewed in Kaltenboeck *et al*., 2005 and by Reinhold *et al*., 2011). The pathogen also caused bovine mastitis, epididymitis and seminal vesiculitis and was excreted in bull semen (Storz *et al*., 1968; Kaltenboeck *et al*., 1997; RØnshold and Basse, 1981; Wehnert *et al*., 1980). Chlamydial strains from ruminant abortion were formerly classified as serotype 1, biotype 1, immunotype 1, or outer membrane protein A (*ompA*) gene type B577 of ruminant *Chlamydiae* (Schachter *et al*., 1975; Spears and Storz, 1979; Perez-Martinez and Storz, 1985; Kaltenboeck *et al*., 1993). Thus, *C. abortus* in cattle is predominantly associated with genital tract disease and mastitis. Exposure of pregnant women to *C. abortus* infected ruminants can lead to abortion or stillbirth (Buxton, 1986; Villemonteix *et al*., 1990; Hadley *et al*., 1992; Hyde and Benirschke, 1997; Jorgensen, 1997; Kampinga *et al*., 2000; Pospischil *et al*., 2002; Baud *et al*., 2008).

In the former century, a second chlamydial agent was reported to be associated with growth retardation, abortion, sporadic bovine encephalomyelitis, pneumonia, enteritis, polyarthritis, keratoconjunctivitis, nephritis or purulent endometritis in cattle. This chlamydial agent was first identified as serotype 2 of ruminant *Chlamydiae*, biotype 2, immunotype 2 or *ompA* type LW613 (Schachter *et al*., 1975; Spears and Storz, 1979; Perez-Martinez and Storz, 1985; Kaltenboeck *et al*., 1993). However, it became clear that these disease manifestations were actually induced by a serologically and pathologically diverse new species designated *C. pecorum* (Fukushi and Hirai,

1992). This agent is not known to cause disease in humans.

The use of highly specific and sensitive nucleic amplification methods has also identified *C. psittaci*, albeit less frequently (Borel *et al*., 2006; Twomney *et al*., 2006; Kauffold *et al*., 2007; Pantchev *et al*., 2009; Kemmerling *et al*., 2009), and rarely *C. suis* in cattle (Teankum *et al*., 2007; Pantchev *et al*., 2009). Birds and swine are respectively, the main hosts for these two species. *C. psittaci* causes reproductive failure and respiratory disease in cattle, while the clinical significance of the occurrence of *C. suis* in cattle remains unclear.

Chlamydiaceae infections in sheep and goats are caused by *C. abortus* and *C. pecorum*. Despite the existence of commercially available vaccines, *C. abortus* induced lamb loss and enzootic abortion during the last third week of gestation still remains the most common cause of reproductive failure in sheep breeding countries all over the world. *C. abortus* causes major economic losses in affected flocks. Enzootic abortion in ewes (OEA) is a notifiable disease in Belgium and is also notifiable to the World Organisation for Animal Health (OIE). *C. pecorum* occasionally causes abortion in small ruminants. Especially in lambs, *C. pecorum* can induce, according to the subtype, pneumonia, polyarthritis, conjunctivitis, enteritis or clinically inapparent infections (Rodolakis *et al*., 1998; Berri *et al*., 2009).

The present study examines the occurrence of *C. abortus* on Belgian sheep, goat and cattle farms, as epidemiological data on this zoonotic pathogen are lacking.

2. Materials and Methods

2.1. Transversal sero-epidemiological study in small and large ruminants

Sheep (n=988), goat (n=48) and cattle (n=1887) sera were provided by the biobanks of the following organisations: CODA-CERVA (Veterinary and Agrochemical Research Centre, Brussels), ARSIA (Association Régionale de Santé et d'Identification Animales, Ciney) and DGZ (Dierengezondheidszorg Vlaanderen, Drongen). For sheep and cattle, examined herds were proportional to the number of herds in each Belgian Province. The survey included 98 sheep herds from the list of volunteers enrolled in the Visna-Maedi certification program. For cattle herds (n=133), samples originated from the whole Belgian population. In addition, available sera of 9

goat herds were serologically examined. Available sera of cattle were limited to animals ageing at least 24 months, while sheep and goat sera were not restricted by age category. None of the examined animals were vaccinated against *C. abortus*.

Sample collection and preparation was as follows: blood was collected by vene puncture (*v. jugularis*) during the winter of 2009-2010, incubated overnight at room temperature, centrifugated (325 g, 4 ℃, 10 min) and serum collected and stored at -20 ℃. All sera were tested for the presence of antibodies against *C. abortus* using the same batch of the ID Screen® *Chlamydia abortus* indirect multi-species ELISA (IDVET Innovative Diagnostics, Montpellier, France), which is currently the only commercially available *C. abortus* ELISA. This ELISA uses microwells coated with a synthetic peptide from the major outer membrane protein (MOMP) specific for *C. abortus*. For small ruminants, the specificity of the test is up to 99.5%, while the sensitivity is expected to be 80% (difficult to find a large infected population) (P. Pourquier, personal communication, 2012). However, in cattle, the sensitivity is even more difficult to settle, because of very low confirmed *C. abortus* cases in cattle (P. Pourquier, personal communication, 2012). The assay was performed according to the instructions of the manufacturer. Samples were tested at a dilution of 1/100. For each sample, the S/P was calculated, which is 100 times the OD_{450} of the sample/mean value of the positive control OD_{450}. Samples presenting an S/P of: i) less than or equal to 50% were considered negative, ii) less than 60% and greater than 50% were considered doubtful and, iii) greater than or equal to 60% were considered positive. A herd was considered positive or suspicious for *C. abortus* antibodies when at least one animal tested positive or doubtful, respectively.

2.2. Molecular diagnosis on 3 cattle farms

During 2012, rectal swabs of 2 Belgian cattle farms in the province of East-Flanders (farms A and B) and one Belgian farm in the province of Limburg (farm C) (20 swabs per farm) were examined for the presence of *Chlamydiaceae*. Animals of different ages and gender were tested. On farm A (meat cattle), only bulls (n = 20) of 12-24 months were sampled while on farm B (meat and dairy cattle), bulls of 12-24 months (n = 5), cows > 24 months (n = 5), heifers (n = 5) and calves between the age of 1 day to 4 months (n = 5) were examined. On farm C (meat and dairy cattle), bulls of 12-24 months (n = 5), cows > 24 months (n = 5) and calves between the age 4 and 12 months (n = 5)

were examined. The farms participated in an EHEC survey (Joris *et al*., 2012) and were also willing to provide samples for *Chlamydia* diagnosis. There was no history of abortion.

All rectal swabs were placed in 2 ml DNA/RNA stabilisation buffer, transported on ice and stored at -80 °C until tested. Molecular diagnosis was performed by the 23S rRNA-based ArrayTube™ (AT) microarray (AlereTechnologies GmbH, Jena, Germany) (Sachse *et al*., 2005). The assay detects all currently known *Chlamydiaceae* species, including *C. abortus*.

2.3. Statistics

Only descriptive statistics were used as available sample size was highly variable between herds. Herd status is separately presented for herds for which sample size was < 10 animals or ≥ 10 animals.

3. Results

3.1. Transversal sero-epidemiological study in sheep

Analysis of 10 to 20 samples per herd, with the exception of 1 herd for which 44 sera were tested, revealed of seropositive and suspicious herd status in 14.3% (6/42) and 11.9% (5/42) of examined herds, respectively. Seropositive or suspicious herds were detected in West Flanders, East Flanders, Hainaut, Namur, Liège and Luxembourg (Figure 1 and 2). Prevalence of seropositive or suspicious animals in said herds was low. For one herd in Luxembourg, 2 of 17 examined sheep tested seropositive. In all other seropositive or suspicious herds, only 1 sampled animal per herd tested positive or suspicious. Only 6 of 280 sheep sera from 56 herds for which sample size at herd level was < 10 animals tested positive, counting for an additional 6 seropositive herds in Belgium. These were 2 herds in East-Flanders (2/18), 2 in Limburg (2/8) and 2 in Hainaut (2/4). None of the low sample size tested herds in West Flanders (n=10), Antwerp (n=6), Walloon Brabant (n=1), Liège (n=5), Luxembourg (n=1) or Namur (n=3) tested seropositive or suspicious for *C. abortus* antibodies. Overall, all examined sera of sheep from Brabant (12 herds, 140 samples) and Antwerp (8 herds, 60 samples) tested seronegative. Herds counting over 50 animals tested all seronegative.

3.2. Transversal sero-epidemiological study in cattle

Analysis of 10 to 20 samples per herd, with the exception of 1 herd for which 27 sera were tested, revealed of seropositive and suspicious herd status in 11.6% (11/95) and 6.3% (6/95) of examined herds, respectively. Seropositive or suspicious herds were detected in all Belgian provinces except for East Flanders, Limburg and Namur (Figure 1 and 2). Prevalence of seropositive or suspicious animals in examined herds was low. In only 5 herds, more than one animal tested positive or suspicious. In one herd in Liège, 20% (4/20) of examined samples was seropositive, an additional 10% (2/20) of samples revealing a doubtful result. In another herd in Liège, 11.8% (2/17) and 5.9% (1/17) of samples tested seropositive and doubtful, respectively. In Luxembourg, one herd also showed a seroprevalence of 11.8% (2/17). Finally, one seropositive herd in West Flanders and one suspicious herd in Hainaut showed a positive and doubtful rate of 10% (2/20) of examined samples, respectively. In all other seropositive or suspicious herds, only 1 sampled animal per herd tested positive or suspicious. None of 158 examined cattle sera of 38 herds for which sample size at herd level was < 10 animals tested positive, but 2 test results were doubtful. One of these was sampled from a herd in East Flanders for which 6 animals were examined and the other originated from a herd in Hainaut for which 8 animals were examined. Low sample size analysis thus revealed a seronegative herd status in 8/9 herds in East Flanders, 3/4 herds in Hainaut and all sampled herds in West Flanders (n=6), Antwerp (n=4), Limburg (n=1), Flemish Brabant (n=5), Walloon Brabant (n=2), Liège (n=4), Luxembourg (n=1) and Namur (n=2). Overall, all examined sera of cattle from Limburg (7 herds, 89 samples) and Namur (7 herds, 106 samples) tested seronegative. All herds of less than 10 animals tested seronegative.

3.3. Transversal sero-epidemiological study in goat

In total 9 goat herds were tested, of which two herds with sample size ≥ 10 and seven herds with sample size <10. Seronegativity at herd level was seen in 6 of 7 (85.7%) low sample size tested herds (Liège: n=2, Waloon Brabant: n=1; Luxembourg: n=1, Hainaut: n=1, West Flanders: n=1). A goat herd in East Flanders for which only one sample was examined tested suspicious. Both higher sample-sized goat herds tested either seropositive or suspicious. A high intra-herd prevalence was seen in the seropositive herd (52.9%; 9/17) located in Luxembourg. A dubious result was found in 1/12 (8.3%) sampled goat from a herd in Hainaut.

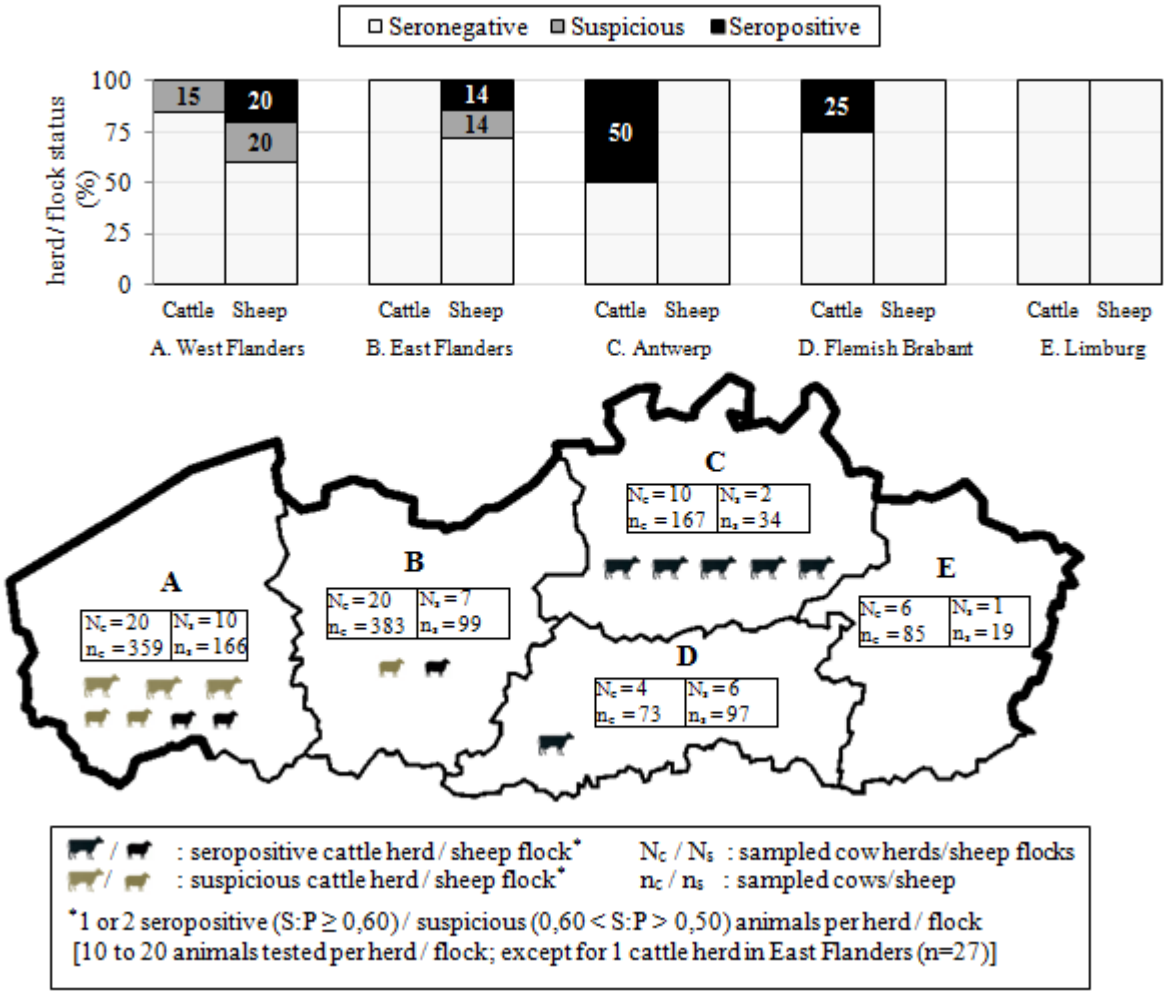

Figure 1. Occurence of *C. abortus* antibodies in Flemish ruminant herds

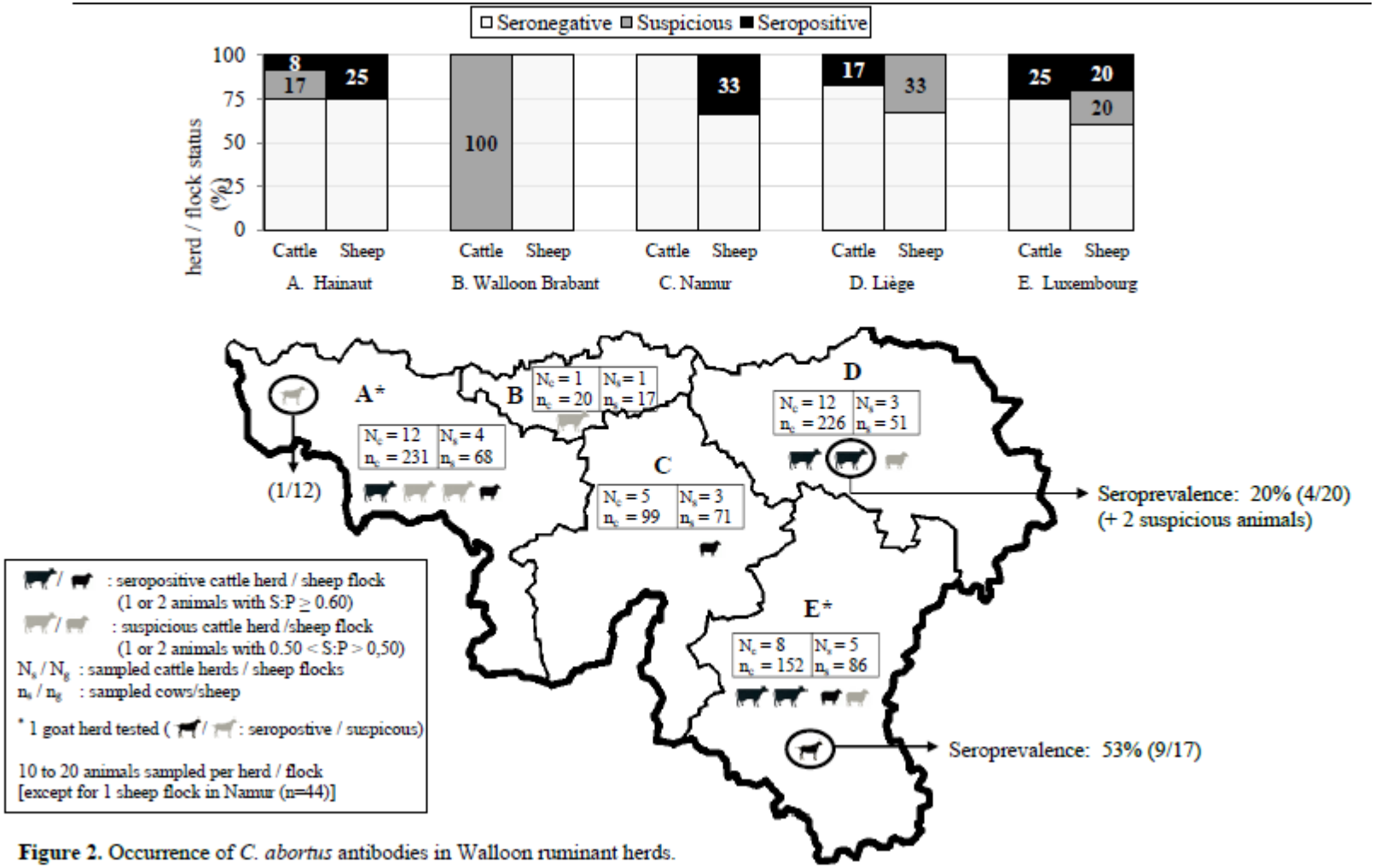

Figure 2. Occurrence of *C. abortus* antibodies in Walloon ruminant herds.

3.4. Molecular diagnosis on 3 cattle farms

All rectal swabs were *Chlamydiaceae* negative when using the ArrayTube™ (AT) microarray.

4. Discussion

C. abortus is frequently isolated from ruminants and is responsible for abortion, infertility, keratoconjunctivitis, pneumonia, enteritis, mastitis and arthritis. Abortus and keratoconjunctivitis have been reporoducted in experimentally infected sheep (Wilsmore *et al*., 1990; Tsakos *et al*., 2001; Navarros *et al*., 2004; Longbottom *et al*., 2013b). Transmission occurs orally or sexually. *C. abortus* is a zoonotic pathogen. Several cases of abortion have been reported in pregnant women after contacted with sheep (Beer *et al*., 1982; Johnson *et al*., 1985; McKinlay *et al*., 1985; Wong *et al*., 1985; Buxton *et al*., 1986, Herring *et al*., 1987; McGivern *et al*., 1988; Jorgensen, 1997, Hyde and Benirschke, 1997; Kampinga *et al*., 2000; Walder *et al*., 2005; Janssen *et al*., 2006) and goats (Pospischil *et al*., 2002; Meijer *et al*., 2004). Human infection is characterized by acute flu-like symptoms followed by abortion and in some cases renal blocking, hepatic dysfunction and extensive intravascular coagulation resulting in death. No information is available on the *C. abortus* infection status in Belgian ruminants or on the risk for human health. We therefore conducted a seroprevalence study in cattle, sheep and goats. We used the ID Screen® *Chlamydia abortus* indirect multi-species ELISA (IDVET Innovative Diagnostics, Montpellier, France) since it is the only commercially available *C. abortus* ELISA with high sensitivity and specificity.

At sample size of 10 or more examined animals per herd, current results reveal a seropositive status in 11.6% (11/95) and suspicious status in 6.3% (6/95) of examined Belgian cattle herds. Seroprevalence in positive herds was relatively low, with generally only 1 or 2 seropositive animals on 10 to 20 tested animals per herd. This may explain a much higher rate of seronegativity at herd level in herds for which < 10 animals were tested. Here, none of 38 examined cattle herds tested positive and only 2 herds (5.3%) were suspicious. Recently, Wilson *et al*., (2012), performed a study in Irish cattle (100 herds, 20 samples/herd) using a soluble chlamydial antigen (detergent treatment of elementary bodies) ELISA (Wilson *et al*.,

2009) detecting antibodies against both *C. abortus* and *C. pecorum* and reported a seroprevalence rate of 6.04% at the animal level. Their results suggested that the prevalence of chlamydial infections was, as for Belgium, low in cattle in Ireland. Still, prevalence at herd level was much higher in Irish herds (60%). The seropositive rate in Belgian cattle herds (11.6% if tested at n ≥10) in the current study could be an underestimation of the real level of infection of cattle in Belgium as the sensitivity of the ID VET ELISA could not yet be tested due to very few confirmed *C. abortus* cases in cattle. This could also explain the apparent absence of infected herds in the provinces of Limburg and Namur. Consequently, we might underestimate the human exposure rate. Seroprevalence studies on cattle in most other countries reported higher infection rates at animal level (reviewed in Reinhold *et al*., 2011). For instance, Wehrend *et al*. (2005) used a genus-specific ELISA. They found a seroprevalence of 41.5% in 445 dairy cows from 34 German farms, which is probably not abnormal as the ELISA detected antibodies against all members of the genus *Chlamydia.* Cavirani *et al*., (2001) examined 671 dairy cows of the Po Valley of northern Italy using a commercial indirect ELISA (CHEKIT, Bommeli AG-IDEXX) and found a prevalence rate of 24.0%. The latter two studies focused only on dairy farms with fertility problems, which could explain higher infection rates compared to Belgian cattle, as this sub-population has probably higher infection levels than the general population. Then again, results of Godin *et al*. (2008) suggest that *C. abortus* is rare in Swedish dairy cows with reproductive disorders. These authors examined 525 cows in 70 Swedish dairy herds by use of both the CHEKIT ELISA and the commercial Pourquier C. *abortus* ELISA (InstitutPourquier, Montpellier, France). The Pourquier ELISA, which uses a recombinant fragment of an 80–90 kDa polymorphic outer membrane protein as antigen, revealed a seroprevalence rate of 0.4% at animal level. The CHECKIT ELISA, however, showed a seroprevalence of 28% (Godin *et al*., 2008). These and other experimental results indicate a lower specificity of the CHECKIT ELISA as compared to the Pourquier ELISA (Vretou *et al*., 2007; Godin *et al*., 2008, Wilson *et al*., 2009). We also decided to use the Pourquier *C. abortus* ELISA for our study. However, it was recently removed from the market. Recentally, a new serological test became available, namely the Chlamydia, HIPPRA CIVT. However, it is sensitively and specificity has not been compared to the other presently available tests.

Serological examination of 10 or more animals per herd revealed a positive herd status in 14.3% (6/42) of Belgian sheep, whereas at lower sample size only 10.6% (6/56) of herds tested seropositive. As for cattle, the occurrence of *C. abortus* antibodies appears low in Belgian sheep as compared to most other reports on seroprevalence rates in non-vaccinated sheep: Switzerland (9.2 to 19.0%) (Borel *et al*., 2004, 2012), Ireland (11%) (Markey *et al*., 1993) and Germany (15.1 to 94.0%) (Lenzko *et al*., 2011; Runge *et al*., 2012). Then again, one of two Belgian goat herds that was examined at sample size ≥ 10 tested seropositive, revealing an intra-herd seroprevalence of 52.9% (9/17). Nevertheless, large variations in reported seroprevalence rates may in part be due to differences in: i) sensitivity and/or specificity of serological tests used, ii) the size of the population under study, iii) differences in ruminant breeds, iv) vaccination status, v) and management (indoor or outdoor housing, import of animals and/or sperm).

It is noteworthy that in the UK, the number of reported cases in sheep was considerably higher in farms holding more than 150 animals (47.6%), as compared to smaller herds (< 150 animals) (9.4%) (Longbottom *et al*., 2013a). We obtained opposite results: no infection in flocks of larger size. Moreover, *C. abortus* morbidity in Belgian sheep seems to be low given the low prevalence in infected herds. This poses questions on the size of the examined population, which was maybe too low. It is therefore possible that the rate of infection in sheep is underestimated. A study on the management conditions and risk factors, as initially planned, could answer these questions. It is therefore regrettable, albeit understandable, that the sector was not very willing to provide an active contribution to this study, as we had difficulties in getting enough sheep serum samples.

Our data suggest a limited infectivity for *C. abortus* in sheep and cattle, as the intra-herd seroprevalence in the positive herds was rather low. Wilson *et al*., (2012) observed the same. In light of current results, further research would be useful to define the actual exposure level for the human population at risk. Seropositive Belgian farms could be used for zoonotic risk analysis. In the future, it would be worthwhile to examine a possible aetiological link between *C. abortus* infection in Belgian ruminants and reproductive disease or conjunctivitis (sheep),

which requires further investigation with samples obtained from herds with clearly documented clinical data.

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Chapter Three

Chlamydia psittaci in chickens

Part A

Pathogenicity of low and highly virulent *Chlamydia psittaci* isolates for specific-pathogen-free chickens

Adapted from:

Lizi Yin, Stefanie Lagae, Isabelle Kalmar, Nicole Borel, Andreas Pospischil, Daisy Vanrompay. Pathogenicity of low and highly virulent *Chlamydia psittaci* isolates for specific-pathogen-free chickens. Avian Diseases. (accepted, 2013)

Abstract

In commercially raised poultry, chlamydiosis mostly seems to occur on turkey or duck farms, sometimes associated with zoonotic transmission and disease (psittacosis) in humans. However, *Chlamydia* infections are apparently emerging in chickens. Information on the virulence of *Chlamydia* in chickens is limited. Up to date, *C. psittaci* genotypes B and D were most frequently found in broilers. We examined the pathogenicity of the well characterized *C. psittaci* genotype B (CP3) and D (92/1293) strains in experimentally (aerosol) infected SPF chickens. Both strains caused conjunctivitis, rhinitis and dyspnoea. Pharyngeal and cloacal *C. psittaci* excretions were observed in all infected animals, indicative for systemic dissemination as proven by immunofluorescence staining of frozen tissue sections. Histopathological lesions were present in all infected chickens. However, differences in pathology were observed as genotype D was more virulent than genotype B, creating mortality and more severe clinical signs and lesions.

Keywords: *Chlamydia psittaci*, chickens, poultry, broilers, pathology

1. Introduction

C. psittaci is an obligate intracellular, Gram-negative bacterium causing respiratory disease in poultry. Avian *C. psittaci* is classified into the well-characterized outer membrane protein A (*ompA*) genotypes A-F and E/B. Genotypes B, C, D, F and E/B have been found in chickens (Vanrompay *et al*., 1997; Gaede *et al*., 2008; Zhang *et al*., 2008; Dickx *et al*., 2010; Zhou *et al*., 2010; Yin *et al*., 2013).

In commercially raised poultry, chlamydiosis mostly seems to occur on turkey and duck farms, sometimes associated with zoonotic transmission and disease (psittacosis) in humans. However, *Chlamydia* infections are apparently emerging in chickens. Dickx *et al*., (2010) examined 10 randomly selected Belgian broiler breeder, broiler and layer farms by a recombinant MOMP-based antibody ELISA (Verminnen *et al*., 2006) and found 98, 95, and 95% seropositive layers, broilers, and broiler breeders, respectively. Moreover, they demonstrated the presence of *C. psittaci* genotype D strains in the air of chicken hatching chambers and in Belgian and French broilers sampled at slaughter. Zoonotic transmission of genotype D strains to hatchery and abattoir employees did occurred (Dickx *et al*., 2010, 2011). In France, 3 cases of atypical pneumonia in individuals working at a French poultry slaughterhouse that processes guinea fowl, ducks and especially chickens (Laroucau *et al*., 2009), prompted Laroucau *et al*., (2009) to conduct an epidemiological survey in 10 supplying poultry farms. Using a *Chlamydiaceae*-specific real-time PCR, chlamydial agents were detected in 12 of 18 (67%) investigated chicken flocks. Positivity for the flocks ranged between 10 and 100%. Rather unexpected, ArrayTube DNA microarray testing for further characterization of the chlamydial agents indicated the presence of an atypical chlamydia agent in 7 chicken flocks, originating from 6 different breeders. Surprisingly, all chicken flocks appeared healthy. Recent data suggest that these new chlamydial agent could putatively be widespread in Australian, France, Greek, Croatian, Slovenian and Chinese chicken flocks (Roberson *et al*., 2010; Zocevic *et al*., 2012).

Information on the virulence of *Chlamydia* in chickens is limited. Up to date, *C. psittaci* genotypes B and D were most frequently found in broilers (Vanrompay *et al*., 1997; Dickx *et*

al., 2011; Yin *et al*., 2013). Beeckman *et al*., (2010) already performed a study in chicken macrophages (HD11 cells) comparing host pathogen interactions of the low virulent genotype B reference strain CP3 (Bankowski and Page, 1959; Piraino, 1969) to the highly virulent genotype D strain (92/1293). CP3 was isolated in 1957 from a Californian pigeon (Bankowski and Page, 1959), while 92/1293 was isolated in 1992 from Dutch diseased turkeys (Vanrompay *et al*., 1993). The genotype D strain: 1) clearly induced actin recruitment to the site of chlamydia entry and invaded the host cells more efficiently, 2) initiated host cell degeneration at earlier time points, and 3) survived and proliferated better in macrophages when compared to the low virulent CP3 strain.

The purpose of the present research was to study the pathogenicity of CP3 and 92/1293 *in vivo* in specific-pathogen-free (SPF) chickens and to compare the results with the previously obtained *in vitro* (HD11 cells) data for the pathogenicity of this pathogen (Beeckman *et al*., 2010), which according to recent literature seems to be emerging in chickens.

2. Materials and Methods

2.1. *Chlamydia psittaci*

C. psittaci genotype D strain 92/1293 (Vanrompay *et al*., 1993) and *C. psittaci* genotype B strain CP3 (ATCC VR-574) (Bankowski and Page, 1959) were used. *C. psittaci* was grown in Buffalo Green Monkey (BGM) cells as previously described (Vanrompay *et al*., 1992). Bacterial titration was performed by the method of Spearman and Kaerber (Mayr *et al*., 1974) to determine the 50% tissue culture infective dose ($TCID_{50}$) per ml.

2.2. Experimental infection

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University (EC 2010/054). Briefly, 3 groups of 22 day-old SPF chickens (Lohman, Cuxhaven, Germany) were individually tagged and housed in separate negative pressure isolators (IM1500, Montair, Sevenum, The Netherlands). At the age one week, groups 1 and 2 were exposed for 1 h to an aerosol containing 10^6 $TCID_{50}$ of *C. psittaci* genotype B (CP3) or genotype D (92/1293) suspended in PBS (5 µm droplets; Cirrus™ nebulizer; Lameris,

Aartselaar, Belgium), respectively. A third group received an aerosol of PBS and served as non-infected control.

2.2. Clinical signs and macroscopic lesions

Clinical signs were daily recorded until 34 days post infection (dpi). Clinical signs were scored. Clinical score 0 indicated no clinical signs; score (1) conjunctivitis; score (2) rhinitis; score (3) conjunctivitis and rhinitis; score (4) dyspnoea; score (5) conjunctivitis, rhinitis and dyspnoea; score (6) green watery droppings, conjunctivitis, rhinitis and dyspnoea. It was our purpose to euthanize two birds per group at 2, 4, 6, 8, 10, 14, 17, 21, 24, 28 and 34 dpi for detailed examination. However, dead birds would be examined immediately regardless the dpi. Macroscopic lesions were scored according to Table 1.

2.3. *C. psittaci* excretion

Pharyngeal and cloacal excretion were determined by examining rayon-tipped, aluminium-shafted swabs (Colpan; Fiers, Kuurne, Belgium) provided with *C. psittaci* transport medium and used for sampling at euthanasia. Swabs were stored at -80 ℃ until processed. *Chlamydia* excretion was monitored using standard procedures for culture, and bacterial identification (IMAGEN^{TM} immunofluorescence staining). The presence of *Chlamydia* was enumerated in five randomly selected microscopic fields (600X, Nikon Eclipse TE2000-E, Japan) and results were scored from 0 to 5. Score 0 indicated that no *C. psittaci* was present; score 1 was given when a mean of 1-5 elementary bodies was present; scores 2, 3, 4 and 5 were given when a mean of 1-5, 6-10, 11-20 and > 20 inclusion positive cells was present.

Table 1. Macroscopic lesion scoring system

Tissue	**Lesion score 1**	**Lesion score 2**	**Lesion score 3**
Conjunctiva	Congestion unilateral	Moderate congestion bilateral	Severe congestion bilateral
Conchae	Slightly congested	Severely congested	Severely congested + viscous mucus
Lung	Congestion bilateral	Congestion + grey foci unilateral	Congestion + grey foci bilateral
Thoracic air sac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Abdominal air sac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Pericardium	Serous pericarditis	Serous pericarditis	Serous adhesive pericarditis
Spleen	Slightly enlarged	Moderately enlarged	Severely enlarged + petechiae
Liver	Slightly congested	Moderately congested	Moderately congested + petechiae
Kidney	Slightly enlarged	Moderately enlarged	Severely enlarged
Intestine	Slightly congested	Moderately congested and fluid inside	Severely congested and fluid inside

Tissues with no lesions were scored 0

2.4. *C. psittaci* replication in tissues

At euthanasia, tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic air sacs, pericardium, spleen, liver, kidney and jejunum were immersed in methocel (Methocel MC, Sigma), snap frozen in liquid nitrogen and stored at -80 ℃ until processed. Cryostat tissue sections (5 µm) were examined for the presence of *Chlamydia* by the IMAGEN™ immunofluorescence staining. The presence of *Chlamydia* was enumerated as for chlamydial excretion.

2.5. Immunohistochemistry and histopathology

At euthanasia, tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic air sacs, pericardium, spleen, liver, kidney, jejunum and ovary/testis were fixed in formalin (4% buffered neutral formaldehyde), processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin for histopathology. For immunohistochemistry fixed samples were dehydrated after 24 hours and embedded in paraffin. Sections were stained with a *Chlamydiaceae* family-specific mouse monoclonal antibody (mAb) directed against the chlamydial lipopolysaccharide (LPS) (mLPS; clone ACI-P; Progen Biotechnik GmbH, Heidelberg, Germany). All slides were examined microscopically (Leitz, New York, USA). Histopathological findings were graded [mild (1), moderate (2), or severe (3)], while immunohistochemical findings were scored from 1 to 4, depending on the number of positive cells observed.

2.6. Statistics

Means of 2 birds per sample point (dpi) were pooled per infection group (CP3, 92/1293 or negative control) for the entire course of infection as monitored from 2 dpi until 34 dpi, and into one of three stages of infection: early infection (2, 4 and 6 dpi), mid infection (8, 10, 14 and 17 dpi) and late infection (21, 24, 28 and 34 dpi). Data were subject to the nonparametric Kruskal-Wallis one way ANOVA test using SPSS® Statistics version 21 (IBM®, Somers, NY). Significance was set at $p < 0.050$.

3. Results

3.1. Clinical signs and macroscopic lesions

Non-infected controls (group 3) remained healthy throughout the experiment. All chickens in groups 1 and 2 showed respiratory disease. However, clinical signs were more severe in chickens infected with the highly virulent genotype D strain 92/1293 (group 2). Early infection with the genotype D strain was characterized by respiratory symptoms, which were exacerbated during mid infection and diminished but not completely resolved during late infection. Anorexia and mortality were only observed during mid infection. Respiratory symptoms in this group were first observed at 3 dpi, when 4 of 20 remaining chickens showed conjunctivitis (scratched their eyes), and rhinitis (head shaking). Chickens of group 2 showed conjunctivitis, rhinitis, dyspnoea and watery droppings, being present in all chickens at 8 dpi. Symptoms were most severe from 8 to 17 dpi. At that time all chickens showed anorexia and they were gasping and sitting down with drooping wings. Afterwards, only slight dyspnoea (open beak breathing) and occasionally head shaking (rhinitis) could be observed until the end of the experiment at 34 dpi. Two chickens died (one at 8 dpi and the other at 9 dpi).

Watery droppings, anorexia or mortality were not observed in genotype B infected chickens. Respiratory symptoms due to a genotype B infection were less severe as compared to a genotype D infection, but also started with low incidence and severity during early infection, exacerbated during mid infection and diminished during late infection. Clinical signs first appeared at 4 dpi, when 2 of 20 animals showed conjunctivitis, rhinitis and slight dyspnoea. Conjunctivitis, rhinitis and moderate dyspnoea were observed in all from 10 to 14 dpi. Afterwards, only moderate dyspnoea was observed until 34 dpi. Mortality was not observed.

3.2. *C. psittaci* excretion

Non-infected controls shed no *C. psittaci*. Pharyngeal and cloacal shedding started for both strains at 2 dpi and 6 dpi, but was most pronounced in chickens infected with the highly virulent genotype D strain 92/1293 ($p<0.05$). Besides this overall effect, cloacal excretion was specifically during mid infection significantly higher in genotype D than in genotype B infected

chickens. In addition, a genotype D infection resulted in a significantly higher cloacal excretion during mid infection as compared to early or late infection stages (Table 2).

3.3. *C. psittaci* replication in tissues

C. psittaci was absent in tissues of the control group 3. Overall, infection with strain 92/1293 resulted in significantly higher chlamydial replication in the upper (conchae and trachea) and lower (lung, abdominal and thoracal air sacs) respiratory tract, as well as in the spleen and kidney. Chlamydial replication during mid infection was also more pronounced ($p<0.05$) in conchae, sinus, trachea, abdominal and thoracal air sacs and spleen of strain 92/1293 compared to strain CP3 infected chickens. The same was observed in lung, thoracal air sac and jejunum during late infection (Table 2). From 4 to 21 dpi, all euthanized chickens of group 2 (strain 92/1293) contained *C. psittaci* throughout the upper and lower respiratory tract, whereas the upper and lower respiratory tract of animals of group 1 (strain CP3) was only completely positive on 8 and 10 dpi. Thus, 92/1293 replicated more intensively in the respiratory tract than CP3. Systemic dissemination of the infection was also more pronounced for group 2. For group 2, all examined tissues of chickens euthanized on days 8, 10, 14, and 21 p.i. were positive. For group 1, at 10 dpi, was the only one whereupon all examined tissues were positive for both euthanized chickens. Strain 92/1293 was thus more virulent than strain CP3, as 92/1293 replicated more intensively and during a longer period in several of the examined tissues.

Table 2. Mean culture scores for pharyngeal and cloacal *C. psittaci* excretion and its presence in tissues in early, mid and late infection stage of *C. psittaci* infected chickens.

Infection stage	Group 1 (CP3)				Group 2 (92/1293)			
	early	**mid**	**late**	**overall**	**early**	**mid**	**late**	**overall**
Pharynx	1.2	1.6	0.8	**1.2^{a}**	2.2	2.5	1.0	**1.9^{b}**
Cloacal	0.2	1.5^{a}	0.6	**0.8^{a}**	0.5^{x}	2.4^{b,y}	2.0^{x,y}	**1.7^{b}**
Conjunctiva	0.3	0.5	0.1	**0.3**	0.7^{x,y}	1.3^{x}	0.3^{y}	**0.7**
Conchae	0.2	0.4^{a}	0.1	**0.2^{a}**	0.7	1.5^{b}	0.9	**1.0^{b}**
Sinus	1.0	0.4^{a}	0.3	**0.5**	1.0	1.4^{b}	0.5	**1.0**
Trachea	0.7	0.4^{a}	0.3	**0.4^{a}**	2.0	2.0^{b}	0.8	**1.5^{b}**
Lung	0.5	1.0	0.5^{a}	**0.7^{a}**	1.5	1.9	1.0^{b}	**1.5^{b}**
Thoracal air sac	0.7	1.0^{a}	0.4^{a}	**0.7^{a}**	1.7	2.9^{b}	1.9^{b}	**2.2^{b}**
Abdominal air sac	0.2^{x}	1.3^{a,y}	0.6^{xy}	**0.7^{a}**	1.5	2.8^{b}	1.6	**2.0^{b}**
Pericardium	0.3	0.4	0.1	**0.3**	0.5	1.3	0.9	**0.9**
Spleen	0.2	0.8^{a}	0.8	**0.6^{a}**	0.8	2.4^{b}	1.3	**1.5^{b}**
Liver	0.0	0.9	0.8	**0.6**	0.3	1.6	1.3	**1.1**
Kidney	0.5	0.6	0.0	**0.4^{a}**	1.2	1.8	0.4	**1.1^{b}**
Jejunum	0.0^{x}	0.8^{y}	0.5^{a,xy}	**0.5**	0.0^{x}	1.4^{y}	1.4^{b,y}	**1.0**

early infection: mean scores of 2 birds on 2, 4 and 6 days post infection (dpi)

mid infection: mean scores of 2 birds on 8, 10, 14 and 17 dpi

late infection: mean scores of 2 birds on 21, 24, 28 and 34 dpi

^{a,b}different superscripts within a row indicate a significant difference between same stages of infection (early, mid or late) at $P<0.05$

^{x,y} different superscripts within a row indicate a significant difference between stages of infection (early, mid or late) at $P<0.05$

3.4. Immunohistochemistry and histopathology

Group 1 and 3 were negative by immunohistochemistry. Thus, chlamydial LPS was only found in strain 92/1293 infected chickens, where it could be detected from 4 dpi until 34 dpi onwards. In this group, chlamydial LPS was demonstrated in all examined tissues, except for conjunctiva and jejunum. Its presence was most pronounced during mid infection and was highest in air sacs ($p>0.05$) (Table 3). Although immunohistochemistry revealed systemic dissemination of strain 92/1293, scores and tissue tropism seemed less pronounced as demonstrated by the immunofluorescence stainings on cryosections. The highest scores were found on 10 dpi, which is in accordance with the results of the immunofluorescence staining on frozen tissue sections and of histopathological lesions, which were also most severe on 10 dpi.

Table 3. Mean score for the immunohistochemical detection of chlamydial lipopolysaccharide (LPS) in tissues of chickens infected with genotype D strain 92/1293 at early, mid and late stage of infection. (Chlamydial LPS was not detected in in tissues of chickens infected with genotype B strain CP3).

Infection stage	**early**	**mid**	**late**	**overall**
Conjunctiva	0.0	0.0	0.0	**0.0**
Conchae	0.0	0.3	0.0	**0.1**
Sinus	0.0	0.3	0.0	**0.1**
Trachea	0.2	0.7	0.0	**0.3**
Lung	0.2	0.5	0.0	**0.2**
Thoracal air sac	0.5	2.2	0.5	**1.1**
Abdominal air sac	0.7	3.0	1.5	**1.7**
Pericardium	0.0	0.3	0.2	**0.2**
Spleen	0.0	0.7	0.2	**0.3**
Liver	0.0	0.7	0.0	**0.2**
Kidney	0.0	0.7	0.3	**0.3**
Jejunum	0.0	0.0	0.0	**0.0**
Ovary/testes	0.0	0.5	0.0	**0.2**

early infection: mean scores of 2 birds on 2, 4 and 6 days post infection (dpi)
mid infection: mean scores of 2 birds on 8, 10, 14 and 17 dpi
late infection: mean scores of 2 birds on 21, 24, 28 and 34 dpi

Hematoxylin-eosin staining revealed no lesions in tissues of the control group (Figure 1). Likewise symptomatology and chlamydial replication, histopathological changes were most pronounced in strain 92/1293 infected chickens. Figure 2 illustrates pathological changes observed in the lungs of chickens infected with strain 92/1293 and strain CP3 during early and late infection. Overall, infection with strain 92/1293 resulted in more severe lesions in air sacs, pericardium, kidney and liver compared to infection with strain CP3 ($p<0.05$). Significantly higher lesion scores due to infection with strain 92/1293 were also observed in kidney tissue during early infection, in trachea, air sacs, pericardium and liver during mid infection and in spleen and pericardium during late infection (Table 4).

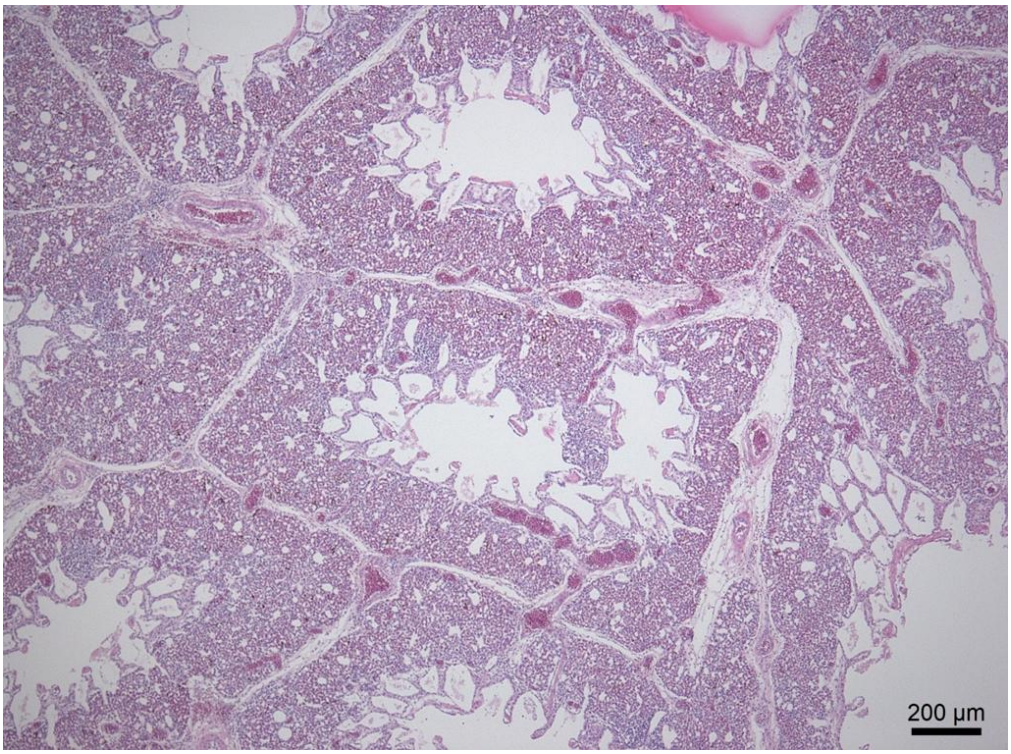

Figure 1. Hematoxylin and eosin staining of chicken lung of an uninfected SPF chicken (10X, 10dpi)

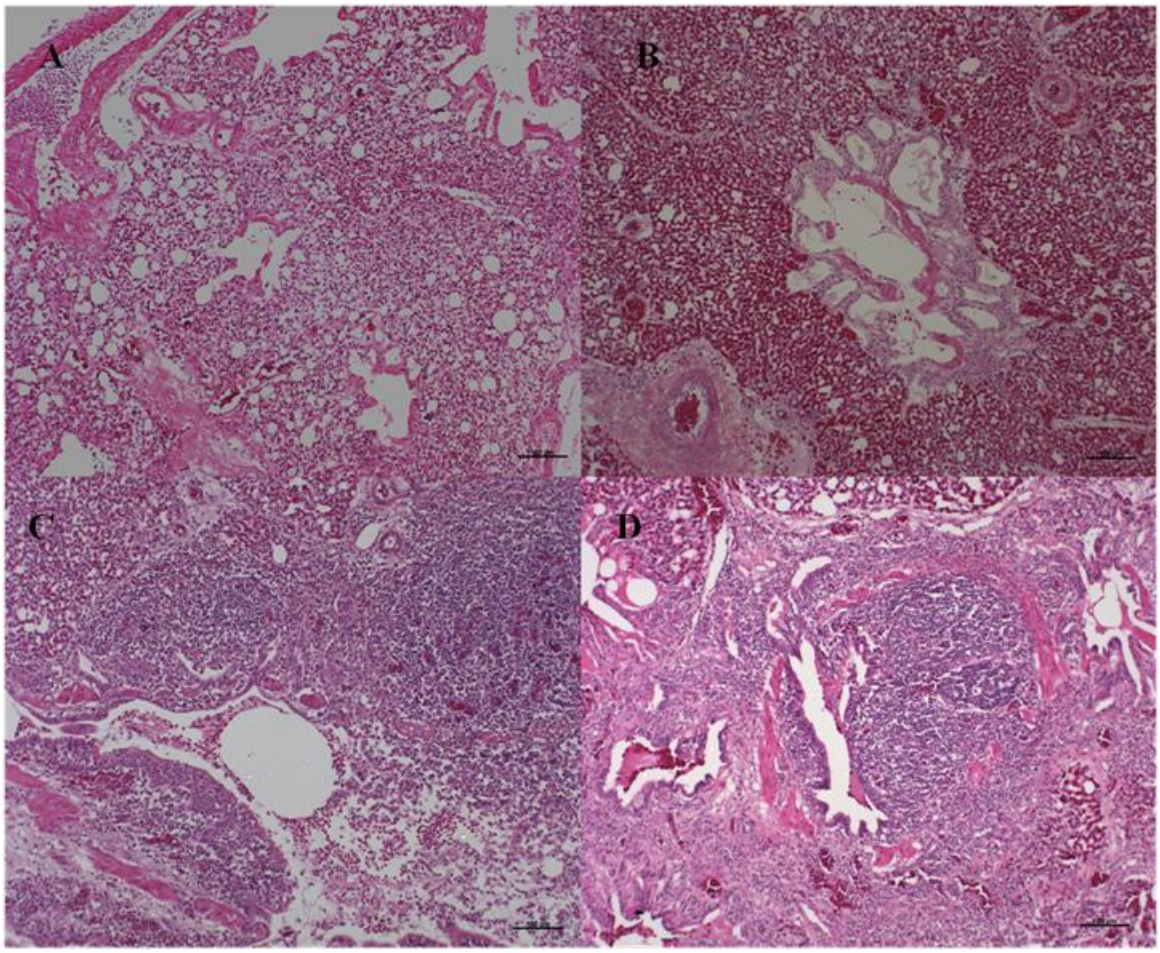

Figure 2. Hematoxylin and eosin staining of chicken lungs infected with *C. psittaci* genotype D strain 92/1293 and genotype B strain CP3 at 6 and 21 days post infection (dpi) (10X).Lungs of chickens infected with *C. psittaci* genotype B show moderate congestion (A) at 6 dpi and diffuse, severe congestion at 21 dpi (B). Infection with *C. psittaci* genotype D results in severe inflammation, lympho-histolytic and heterophilic infiltration at 6 dpi (C) and in severe congestion, BALT hyperplasia, multifocal bronchitis and parabronchitis with lympho-histiocytic in heterophilic infiltration (D).

In general, histopathological lesions in group 1 and 2 were most severe on day 34 and 10 p.i., For group 1, the following was noticed on 34 dpi: 1) Focally, extensive lymphocytic infiltration of the conjunctiva with formation of lympoid nodules, 2) focally, extensive lymphocytic infiltration of the conchae, 3) focal lymphoid epithelial infiltrate in the trachea, 4) fibrous thickening and multifocal lympho-histiocytic infiltration of the abdominal air sac, 5) severe congestion of the lungs with BALT hyperplasia, 6) multifocal lymphocytic infiltration of the liver, and 7) moderate congestion of the spleen.

Table 4. Histopathological lesions* in early, mid and late infection stage of *C. psittaci* infected chickens.

Infection stage	Group 1 (CP3)				Group 2 (92/1293)			
	early	**mid**	**late**	**overall**	**early**	**mid**	**late**	**overall**
Conjunctiva	0.8	3.0	2.3	**2.1**	1.2	1.7	1.8	**1.6**
Conchae	0.7	0.3	0.5	**0.5**	1.3	1.0	0.3	**0.9**
Sinus	0.7	0.0	0.0	**0.2**	1.0	0.0	0.0	**0.3**
Trachea	0.0	0.0[a]	0.2	**0.1**	0.8	2.0[b]	0.0	**0.9**
Lung	2.7	2.0	2.8	**2.5**	2.8	3.0	2.7	**2.8**
Thoracal air sac	0.7	0.3[a]	0.0[a]	**0.3[a]**	1.5	2.8[b]	3.0[b]	**2.4[b]**
Abdominal air sac	0.7	0.5[a]	1.0	**0.7[a]**	1.3	2.7[b]	3.0	**2.3[b]**
Pericardium	0.0	0.3[a]	0.0[a]	**0.1[a]**	0.7	2.2[b]	2.0[b]	**1.6[b]**
Spleen	1.2	1.5	1.3[a]	**1.3**	1.3	2.2	2.0[b]	**1.8**
Liver	0.7	1.0a	0.8	**0.8[a]**	0.8	2.5[b]	2.3	**1.9[b]**
Kidney	0.8[a]	0.7	1.2	**0.9**	2.0[b]	1.8	1.3	**1.7**
Jejunum	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	**0.0**
Ovary/testes	0.0	0.0	0.0	**0.0**	0.0	0.7	0.0	**0.2**

*Histopathological lesion scores: 1 = mild, 2 = moderate, 3 = severe.
early infection: mean scores of 2 birds on 2, 4 and 6 days post infection (dpi)
mid infection: mean scores of 2 birds on 8, 10, 14 and 17 dpi
late infection: mean scores of 2 birds on 21, 24, 28 and 34 dpi
[a,b] different superscripts within a row indicate a significant difference between same stages of infection (early, mid or late) at $P<0.05$

For group 2, the following was observed on 10 dpi: 1) moderate lympho-histiocytic infiltration of the conjunctiva, 2) mild epithelial hyperplasia of the trachea with heterophylic, lympho-histiocytic infiltration, 3) diffuse, severe, fibrinous, heterophylic, histiocytic infammation of the thoracal air sac, 4) moderate to severe proliferative, lympho-histiocytic, heterophylic, fibrinous necrotizing inflammation of the abdominal air sac, 5) congestion of the lungs with moderate to severe pneumonia and peribronchitis (hsitiocytic, heterophylic), 6) pericarditis with diffuse severe heterophylic, lympho-histiocytic infiltration, 7) severe congestion of the liver with multifocal degenerative to necrotizing hepatitis with

lympho-histiocytic infiltration, 8) congestion of the spleen with multifocal severe necrotizing fibrinous splenitis, and 9) diffuse lympho-histiocytic infiltration of the ovaries.

4. Discussion

Avian *C. psittaci* is a risk class 3 pathogen, requiring biosafety level 3 in laboratories willing to isolate the bacterium. Thus, diagnosis of *C. psittaci* in poultry is technically and financially more demanding than diagnosis of other respiratory pathogens. The introduction of nucleic acid amplifications techniques made *C. psittaci* diagnosis more feasible for veterinary clinical laboratories. However, *C. psittaci* diagnosis is for an understandable reason, still not yet routine in veterinary diagnosis. One of the reasons is probably also the fast increasing morbidity and mortality during respiratory disease in poultry. Therefore, antibiotics, often tetracyclines or quinolones, are immediately applied and diagnosis is often no longer relevant. This might be one of the reasons why *C. psittaci* infections are still neglected and thus underestimated in the poultry industry and why respiratory disease still occurs, notwithstanding intensive (viral) vaccination (infectious bronchitis, Newcastle disease virus, avian metapneumovirus) against respiratory disease, as we know that *C. psittaci* interacts with other respiratory pathogens like for instance avian *Escherichia coli* and *Ornithobacterium rhinotracheale* (Van Loock *et al*., 2005, 2006).

The past five years, researchers described the occurrence of *C. psittaci* and atypical *Chlamydia* in chickens (Yang *et al*., 2007; Gaede *et al*., 2008; Zhang *et al*., 2008; Laroucau *et al*., 2009; Robertson *et al*., 2010; Zhou *et al*., 2010; Yin *et al*., 2013). Thus, *Chlamydia* infections seem to be (re)-emerging in Australian, Chinese and European broilers and to a lesser extent in layers, although poultry farmers mostly do not seem to be aware. Some of these papers document clinical disease in chickens (Yang *et al*., 2007; Gaede *et al*., 2008; Zhang *et al*., 2008; Zhou *et al*., 2010; Yin *et al*., 2013) while others report no clinical disease in chickens (Laroucau *et al*., 2009; Robertson *et al*., 2010) but zoonotic transfer and pneumonia in humans (Laroucau *et al*., 2009). Thus, less is known on pathogenicity of *C. psittaci* strains in chickens. *C. psittaci ompA* genotypes B and D often seem to infect chickens (Vanrompay *et al*., 1997; Dickx *et al*., 2010, 2011). This is why we conducted this study, examining the pathogenicity of a well

characterized *C. psittaci* genotype B (CP3) and D (92/1293) strains in experimentally infected SPF chickens.

Both strains caused a clinical course of infection, which started with a low incidence and severity of respiratory symptoms during early infection, exacerbated during mid infection to all infected chickens and diminished again in severity and incidence during late infection. However, respiratory symptoms were more severe in genotype D infected chickens. Moreover, genotype D also caused anorexia and mortality during mid infection, which was not observed in genotype B infected chickens. Pharyngeal and cloacal *C. psittaci* excretion was observed in all infected animals, indicative for systemic dissemination as proven by immunofluorescence staining of frozen tissue sections. Cloacal excretion in genotype D infected chickens was significantly higher during mid infection as compared to early or late infection stages. Strangely, immunohistochemistry could not prove the occurrence of a systemic infection in CP3 infected chickens. Probably, the technique is less sensitive than immunofluorescence staining and/or the LPS of CP3 is no longer detectable after formalin fixation. Nevertheless, histopathological lesions were present in all infected chickens and observations on the pathogenicity were in accordance with those observed during other experimental infections in chickens (Bankowski *et al*., 1967, 1968; Takahashi *et al*., 1988a, 1988b; Suwa *et al*., 1990).

We could only find 5 other reports on experimental infections in chickens. Strains from the following birds have been used: 1) a budgerigar (Izawa-1; genotype A), 2) a parrot (GCP-1; no genotype specified), 3) a pigeon (P-1041; no genotype specified) (Takahashi *et al*., 1988a, 1988b), 4) turkeys (strain C-1; no strain or genotype specified and the Turkey/California/181 strain; no genotype specified) (Bankowski *et al*., 1967, 1968; Banks *et al*., 1970; Suwa *et al*., 1990), and 5) ruminant strains (B-577, Bo-Yokohama and SPV-789) (Takahashi *et al*., 1988b). All these former reports did not use the natural route of infection, namely inhalation of aerosols. *Chlamydiae* were directly injected into the air sac or trachea, or chickens were infected orally. The avian strains (10^5 ELD_{50}) used by Takahashi *et al*. (1988b), induced a generalized infection within 10 dpi followed by death in 8-day-old White Leghorn chickens. Strains isolated from *Psittacidae* were more virulent than the one pigeon strain used, as they caused higher mortality

in chickens. Strains derived from ruminants were far less pathogenic to chickens than avian strains.

In the present study, differences in pathology were also observed as genotype D was more virulent than genotype B, creating mortality and more severe clinical signs and lesions in the genotype D infected group. The same has been observed while examining the developmental cycle of these strains in chicken macrophages (Beeckman *et al*., 2010). Interestingly, similar observations have also been made in SPF turkeys experimentally infected with strain 92/1293 or strain 89/1326, also a pigeon derived genotype B strain. The incubation period for the genotype B strain was also longer, maximal replication was delayed, the period during which bacteria were observed in the same tissue was also shorter and tissue tropism also seemed to be less extensive, as compared to an infection with strain 92/1293 (Vanrompay *et al*., 1994, 1995).

At present, we used non-chicken derived *C. psittaci* genotype B and D strains and demonstrated marked pathogenicity, especially for the genotype D strain in experimentally (aerosol) infected SPF chickens. In the future, experiments on the pathogenicity of chicken-derived genotype B and D strains will be conducted.

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Part B

Emerging *Chlamydia psittaci* infections in the chicken industry and pathology of *Chlamydia psittaci* genotype B and D strains in specific-pathogen-free chickens

Adapted from:

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Abstract

Sera of 30 Belgian and 10 Northern French chicken farms were tested by a *C. psittaci* MOMP-based ELISA. Ninety-six percent, 93% and 90% of the Belgian broilers, broiler breeders and layers were seropositive. Ninety-one percent of the French broilers were seropositive. In addition, tissues of 5 Belgian and 5 French broiler farms were examined at slaughter. All French farms ware culture positive while *C. psittaci* was cultured from the lungs of 80% of examined Belgian farms. *C. psittaci* infections are apparently emerging in chickens raised in Belgium and Northern France. We could prove Hill-Evans postulates for chicken-derived *C. psittaci* genotype B and D strains. Chicken-processing plant employees should be considered a risk group for human psittacosis. There is a need for higher awareness and for efficient risk assessment and management of *C. psittaci* infection in chickens as chlamydiosis in broilers seems to be underdiagnosed and infections with highly virulent strains do occur.

Keywords: *Chlamydia psittaci*, chickens, poultry, broilers, pathology

1. Introduction

Chlamydia (C.) psittaci is an obligate intracellular, Gram-negative bacterium causing respiratory disease in poultry. *C. psittaci* is classified into the well-characterized outer membrane protein A (*ompA*) genotypes A-F and E/B. All genotypes are associated with specific bird orders from which they are predominantly isolated (Pannekoek *et al*., 2010). So, far genotypes B, C, F and E/B have been found in chickens (Gaede *et al*., 2008; Zhang *et al*., 2008; Dickx *et al*., 2010; Zhou *et al*., 2010).

In industrial poultry, chlamydiosis mostly seems to occur on turkey or duck farms, sometimes associated with zoonotic transmission and disease (psittacosis) in humans. Genotypes A, D, and C are often involved. Reports on *C. psittaci* outbreaks on chicken farms or reports on zoonotic transmissions linked to contact with *C. psittaci*-infected chickens are extremely rare. It could be the case that chickens seldom become infected and/or that the strains infecting chickens are less virulent, presenting a minor risk for humans. We therefore investigated the occurrence of *C. psittaci* by performing a retrospective study on 300 serum samples collected in 2005 from 10 randomly selected Belgian broiler chicken breeder, broiler and layer farms. We had no information on the clinical status or antibiotic treatment of the chickens at time of sampling. Sera were examined by a recombinant MOMP-based enzyme-linked immunosorbent assay (ELISA) (Verminnen *et al*., 2006). Ninety-eight, 95, and 95% of the examined layers, broilers, and broiler breeders were seropositive (Dickx *et al*., 2010). Seropositive chickens were present on all farms. Thus, *Chlamydia* infections occur frequently in chickens raised in Belgium.

The same appears to be true for France, as illustrated by a study of Laroucau *et al*. (2009). Briefly, in 2008, three cases of atypical pneumonia in individuals working at a French poultry slaughterhouse that processes guinea fowl, ducks and especially chickens prompted Laroucau *et al*., (2009) to conduct an epidemiological survey in 10 supplying poultry farms. Using a *Chlamydiaceae*-specific real-time PCR assay, chlamydial agents were detected in 12 of 18 (67%) investigated chicken flocks. Positivity for the chicken flocks ranged between 10 and

100%. Rather unexpectedly, ArrayTube DNA microarray testing for further characterization of the chlamydial agents indicated the presence of a new member of the genus *Chlamydia* in 7 chicken flocks, originating from 6 different breeders. Surprisingly, all chicken flocks appeared healthy.

Thus, *Chlamydia* infections are apparently common in Belgian and French chickens, but Hill-Evans postulates (Evans, 1976; Fredricks and Relman, 1996) for establishing microbial disease causation remains to be full-filled for the chicken infectious *Chlamydia* strains. This study aims at gathering information on the current epidemiological status of *Chlamydia* infections in Belgian and French (Northern France) industrial chickens. In addition, we examined the pathology of the most frequently found *C. psittaci* genotypes by performing experimental infections in specific-pathogen-free (SPF) chickens. To our knowledge, we are the first to examine the pathology of chicken-derived *C. psittaci* strains in SPF chickens.

2. Materials and Methods

2.1. Prevalence study

2.1.1. *Chlamydia* seroprevalence in broilers, broiler breeders and layers

Six-hundred chickens from 10 randomly chosen Flemish broiler, broiler breeder and layer farms (20 chickens per farm) were examined at slaughter for the presence of serum antibodies against *Chlamydia*. The chickens were randomly selected. Also, 200 chickens from 10 randomly chosen French (Northern France) broiler farms (20 chickens per farm) were randomly selected at slaughter for serological examination. We received no information on the clinical status of the examined farms, or on antibiotic treatments. Blood samples were stored overnight at room temperature. Sera were collected after centrifugation (325 x g, 10 min, 4 ℃) and stored at -20 ℃. Antibody titres were determined by use of a recombinant *C. psittaci* major outer membrane (MOMP)-based antibody ELISA as described by Verminnen *et al*., (2006). The MOMP of *C. psittaci* possesses genus-specific epitopes, allowing the detection of antibodies against all *Chlamydia* species.

2.1.2. Isolation of *Chlamydia* from broilers

We took organ samples from broilers being slaughtered in an abattoir in West-Flanders (Belgium). One hundred and two lungs, 94 livers and 91 spleens from 5 French (Northern France) broiler farms were randomly collected at slaughter. Each organ sample came from a different chicken. Thus, 287 chickens were sampled in total. All farms had experienced respiratory disease (rhinitis, dyspnoea) during the brood with the need for doxycycline treatment. We were unable to obtain detailed information on diagnoses performed, age of the chickens while being sick, dose and duration of treatments.

Additionally, 50 lungs from 5 Flemish broiler farms (10 lungs/farm) were selected at slaughter based on the presence of pneumonia. Farms had experienced respiratory disease (rhinitis, dyspnoea) with the need for doxycycline treatment. Information on age of the chickens while being sick, dose and duration of treatments are presented in Table 1.

Finally, 9 randomly selected Flemish broiler farms were visited. Forty randomly selected chickens per farm were sampled for *Chlamydia* diagnosis using pharyngeal swabs provided with chlamydia transport medium. Information on sickness during the brood, age, dose and duration of treatments are presented in Table 1.

All organ samples and pharyngeal swabs were transported on ice, delivered to the laboratory within 12 h and stored at -80 ℃ until tested. Culture was performed using Buffalo Green Monkey (BGM) cells, identifying the organism by a direct immunofluorescence staining (IMAGEN^{TM}, Oxoid, United Kingdom) at 6 days post-inoculation. *Chlamydia* positive cells were enumerated in five randomly selected microscopic fields (600X, Nikon Eclipse TE2000-E, Japan) and results were scored from 0 to 5. Score 0 indicated that no *C. psittaci* was present; score 1 was given when a mean of 1-5 elementary bodies was present; scores 2, 3, 4 and 5 were given when a mean of 1-5, 6-10, 11-20 and > 20 inclusion positive cells was present.

Table 1. Information on the sampled Belgian broiler farms

Farm Nr.	Animals per barn	Age at sampling	Coccidiostats		Antibiotic treatments
			Starter feed	Grower feed	
A	30,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
B	25,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
C	25,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline)
D	30,000	Slaughter age	Nicarbazin	Salinomycin	Amoxicillin
E	30,000	Slaughter age	Nicarbazin & Narazin	Salinomycin	Amoxicillin
F	42,000	5 weeks	Nicarbazin & Narazin	Salinomycin	None
G	25,000	4 weeks	Nicarbazin & Narazin	Salinomycin	Tylan for 2 days (1 g/ 10 L and 4200 L/day)
H	20,000	6 weeks	Nicarbazin & Narazin	Salinomycin	Oxacillin (beta-lactam) for 3 days (300 g/day)
I	18,000	4 weeks	None	None	Cosumix (sulphatrimethoprim) when needed
J	14,000	4 weeks	Nicarbazin & Salinomycin	Salinomycin	None
K	15,000	6 weeks	Salinomycin	Salinomycin	None
L	30,000	29 days	Nicarbazin & Narazin	None	Amoxicillin (β-lactam) for 3 days (2 g /10 L/day)
M	25,000	6 weeks	Nicarbazin & Narazin	Salinomycin	Doxycyclin (tetracycline) for 4 days (1 kg/day)
N	30,000	29 days	Monensin	Salinomycin	Emdotrim (sulphatrimetoprim) for 3 days (2 L/ 1000 L and 8000 L)

Farms A to E were sampled (lungs) in the slaughterhouse. Farms F to N were visited for sampling (pharyngeal swabs) in the bar

2.1.3. Molecular characterization of *Chlamydia* strains

Species identification of isolates was performed by the 23S rRNA-based ArrayTube DNA microarray (Sachse *et al*., 2005). A sample was considered ‘‘chlamydia-negative’’ when all signal intensities except the internal staining control (biotin marker) were below NI (normalized signal intensity) = 0.07. *Chlamydia psittaci* strains were further characterized by the outer membrane protein A (*ompA*) genotype-specific real-time PCR (Geens *et al*., 2005b), by the *ompA*-based genotyping ArrayTube DNA microarray (Sachse *et al*., 2008) and by *ompA* sequencing (VIB Genetic Service Facility Antwerp, Belgium)using CTU/CTL primers [3’-ATGAAAAAACTCTTGAAATCGG-5’/3'-3’CAAGATTTTCTAGA(T/C)TTCAT(C/T)TTGTT-5’], generating an amplicon of 1,098 bp (Denamur *et al*., 1991) or primers CPsittGenoFor [3’-GCTACGGGTTCCGCTCT-5’] and CPsittGenoRev [3’-TTTGTTGATYTGAATCGAAGC-5’] (Heddema *et al*., 2006) generating an amplicon of 1,041-bp.

2.2. Animal experiment

2.2.1. Experimental design, animals, *Chlamydia* strains

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University (EC 2010/054). Briefly, 4 groups of 22 day-old SPF chickens (Lohman, Cuxhaven, Germany) were individually tagged and housed in separate negative pressure isolators (IM1500, Montair, Sevenum, The Netherlands). At the age one week, groups 1-3 were exposed for 1 h to an aerosol of 10^6 $TCID_{50}$ *C. psittaci* suspended in PBS (5 µm droplets; Cirrus^{TM} nebulizer; Lameris, Aartselaar, Belgium). A fourth group received an aerosol of PBS and served as non-infected control. Groups 1, 2 and 3 were infected with *C. psittaci* genotype B strain 10/423 (from a Belgian broiler showing pneumonia), *C. psittaci* genotype B strain 10/525 (from a Belgian broiler showing pneumonia) and with *C. psittaci* genotype D strain 10/298 (from a French broiler showing pneumonia), respectively. Both Belgian *Chlamydia* strains came from different farms. Molecular characterization of the *Chlamydia* strains is described in detail in the results.

Contaminating organisms in both the lungs and the inocula used to infect the SPF chickens were absent as demonstrated by: i) bacterial isolation attempts (F. Boyen; Laboratory for

Bacteriology and Mycology, Fac Veterinary Medicine, UGhent), ii) a genus-specific PCR for mycoplasma (Lierz *et al*., 2007), iii) a PCR for the avian metapneumo virus (aMPV) subtypes A, B, C and D (Guionie *et al*., 2007), iv) a PCR for the infectious bronchitis virus (IBV) (Jones *et al*., 2011), and v) a PCR for the infectious laryngotracheitis virus (ILTV) (Creelan *et al*., 2006). P. Butaye (CODA, Brussels, Belgium) and H. Nauwynck (Ghent University, Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology) provided a *Mycoplasma gallisepticum*, aMPV, ILTV (U76/1035) and IB (strain M41) control for PCR.

2.2.2. Clinical parameters and macroscopic lesions

Clinical signs were daily recorded until 34 days post infection (dpi). It was our purpose to euthanize two birds per group at 2, 4, 6, 8, 10, 14, 17, 21, 24, 28 and 34 dpi for detailed examination. However, dead birds would be examined immediately regardless the dpi. Macroscopic lesions were scored according to Table 2.

Table 2. Macroscopic lesion scoring system

Tissue	**Lesion score 1**	**Lesion score 2**	**Lesion score 3**
Conjunctiva	Congestion unilateral	Congestion bilateral	Petechiae
Conchae	Slightly congested	Severely congested	Severely congested + viscous mucus
Lung	Congestion bilateral	Congestion + grey foci unilateral	Congestion + grey foci bilateral
Thoracic air sac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Abdominal air sac	Diffuse opacity	Focal fibrinous airsacculitis	Diffuse fibrinous airsacculitis
Pericardium	Serous pericarditis	Serous pericarditis	Serous adhesive pericarditis
Spleen	Slightly enlarged	Moderately enlarged	Severely enlarged + petechiae
Liver	Slightly congested	Moderately congested	Moderately congested + petechiae
Kidney	Slightly enlarged	Moderately enlarged	Severely enlarged
Intestine	Slightly congested	Moderately congested and fluid inside	Severely congested and fluid inside

Tissues with no lesions were scored 0

2.2.3. *C. psittaci* replication in tissues, examination of lungs and histopathology

At euthanasia, tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic air sacs, pericardium, spleen, liver, kidney, jejunum and ovary/testis were immersed in methocel (Methocel MC, Sigma), snap frozen in liquid nitrogen and stored at -80 °C until processed. Deceased birds were also sampled. Cryostat tissue sections (5 µm) were examined for the presence of *Chlamydia* by the IMAGEN^{TM} immunofluorescence staining. The presence of *Chlamydia* was enumerated as for the field samples. A small part of the lungs, taken at 4 and 8 dpi, was examined for the presence of contaminating bacteria and viruses as described above.

The lungs, the thoracic air sac and the spleen of euthanized birds at 4, 6, 10, 14, 21, 24 and 34 dpi were taken for histopathology. They were fixed in 10% phosphate-buffered formalin, processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. All slides were examined microscopically (Leitz, New York, USA). Histopathology was performed by I. Debyser (Covetop; Consultancy in Veterinary and Toxicological Pathology; Edingen, Belgium). Microscopic findings were graded [minimal histological change (1), slight (2), moderate (3), marked (4) or severe (5)].

2.2.4. *Chlamydia* excretion

Pharyngeal and cloacal excretions were determined by examining rayon-tipped, aluminium-shafted swabs (Colpan; Fiers, Kuurne, Belgium) provided with *C. psittaci* transport medium and used for sampling at euthanasia and for sampling deceased birds. Swabs were stored at -80 °C until processed. *Chlamydia* excretion was monitored using standard procedures for culture, bacterial identification (IMAGEN^{TM} immunofluorescence staining) and quantification (scoring system as for the field samples) of bacteria in BGM cells.

3. Results

3.1. Prevalence study

3.1.1. *Chlamydia* seroprevalence in broilers, broiler breeders and layers

All Belgian broiler, broiler breeder and layer farms and all French broiler farms were

seropositive. Ninety-six percent, 93% and 90% of the Belgian broilers, broiler breeders and layers were seropositive. Ninety-one percent of the French broilers were seropositive. The percentage of seropositive chickens on the Belgian broiler, broiler breeder and layer farms was 10% (1 broiler farm), 100% (9 broiler farms), 20% (1 broiler breeder farm), 30% (1 broiler breeder farm), 90% (1 broiler breeder farm), 100% (7 broiler breeder farms), 20% (1 layer farm), 90% (1 layer farm) and 100% (8 layer farms). Antibody titers on the Belgian farms with a high number of seropositive animals (90-100%) (n = 26) ranged from 1:400 till 1:6400 while 1:100 till 1:800 for the 4 remaining farms.

The percentage of seropositive chickens on the French broiler farms was 30% (2 farms), 90% (1 farm) and 100% (7 farms). Antibody titers on the French farms with a high number of seropositive animals (90-100%) (n = 8) ranged from 1:200 till 1:3200 while 1:100 till 1:400 for the 2 remaining farms.

3.1.2. Isolation of *Chlamydia* from broilers

Results of culture are summarized in Table 3. All five (100%) examined French broiler farms were culture positive. Culture positive lungs, spleens and livers were found on all farms. Cultures scores ranged from 1 to 4. Overall, the lung was more often culture positive than the spleen, and positivity rate was lowest in liver samples. Also, culture scores were always the highest for the lungs (max score 4) followed by the spleen (max score 2) and the liver (max score 1), respectively. That is why we only sampled lungs during the following sampling round for examining Belgian broilers. Lungs of 4 of 5 (80%) Belgian broiler farms were culture positive. The culture scores ranged from 1 to 4.

Table 3. Culture results for organs sampled from French and Belgian broilers.

French broilers								Belgian broilers			
Farm	**Farms size [a]**	**Lung**		**Liver**		**Spleen**		**Farm**	**Farms size [a]**	**Lung**	
No.	**No.**	**No.**	**Positives(%)**	**No.**	**Positives(%)**	**No.**	**Positives(%)**	**No.**	**No.**	**No.**	**Positives(%)**
1	25,000	10	5(50)	19	4(21)	14	5(36)	A	60,000	10	2(20)
2	30,000	22	5(23)	16	9(56)	16	8(50)	B	30,000	10	0(0)
3	50,000	30	15(50)	20	2(10)	20	8(40)	C	60,000	10	5(50)
4	40,000	20	12(60)	20	5(25)	20	9(45)	D	25,000	10	8(80)
5	25,000	20	12(60)	19	4(21)	21	6(28)	E	25,000	10	6(60)
Total		**102**	**49(48)**	**94**	**24(25.5)**	**91**	**36(39.5)**			**50**	**20(40)**

[a]Number of broilers per farm

3.1.3. Molecular characterization of *Chlamydia* strains

Only lung isolates were molecularly characterized, as our first attempts to characterize spleen or liver isolates were repeatedly unsuccessful. Culture scores were also higher in the lungs, making molecular characterization more feasible. We could only characterize 50 of 69 (72%) *Chlamydia* isolates. They all reacted with the *C. psittaci* specific probes in the 23S rRNA-based ArrayTube DNA microarray. Forty-five of 50 *C. psittaci* strains could be successfully genotyped by both the genotype-specific real-time PCR and the *ompA*-based ArrayTube DNA microarray. However, the genotype-specific real-time PCR detected one mixed infection, while the microarray did not. PCR amplification for *ompA* sequencing was successful in 35 of 50 *C. psittaci* DNA samples. Molecular characterization revealed the presence of *C. psittaci* genotype B and D in respectively 1 (40%) and 3 (60%) of 5 *Chlamydia* positive French broiler farms. The remaining fifth farm was dealing with a mixed infection, as genotypes B and D were discovered. Genotype B was detected on all 5 *C. psittaci* positive Belgian farms.

3.2. Animal experiment

3.2.1. Molecular characterization of *Chlamydia* strains used

Strains 10/298, 10/423 and 10/525 were all *C. psittaci* according to the 23S rRNA-based ArrayTube DNA microarray. Genotyping identified strains 10/298 and 10/423 as genotype D and B, respectively by all methods used. However, strain 10/525 reacted with the micro array probe for the proposed provisional genotype YP84 (Sachse *et al*., 2008), while the genotype-specific real-time PCR identified this strains as genotype B. Subsequent sequencing of the *ompA* gene using the primers CPsittGenoFor and CPsittGenoRev revealed 100% identity with the *ompA* gene of the *C. psittaci* genotype B reference strain CP3 (pigeon strain), while only 96 and 93% nucleic acid and amino acid homology, respectively, with strain YP84 (parrot strain). Multi Locus Sequence Typing (MLST) was performed for double-checking (performed by Y. Pannekoek; Amsterdam University). MLST revealed exactly the same Sequence Type (Pannekoek *et al*., 2010) as for CP3. Strain 10/525 was therefore assigned as genotype B.

3.2.2. Clinical signs and macroscopic lesions

Non-infected controls remained healthy throughout the experiment. Chickens in groups 1 and 2, which were infected with the genotype B strains, showed slight apathy and conjunctivitis, rhinitis and mild dyspnoea from 4 till 16 dpi and had mild intermittent diarrhea (green watery droppings) from 10 till 16 dpi. The number of affected animals gradually decreased with only 2 of 10 (20%) and 4 of 10 (40%) remaining chickens of groups 1 and 2, respectively, showing clinical signs at 16 dpi. Chickens infected with the genotype D strain (group 3) were severely ill and 11 of 22 (50%) animals died between 7 and 10 dpi. Symptoms started at 2 dpi in 8 of 22 (36%) chickens and from 4 dpi onwards, all animals showed marked apathy and anorexia, severe conjunctivitis and rhinitis, exacerbating dyspnoea (mostly sitting on the floor, eyes closed, heads hanging down, sometimes scratching their eyes, gasping and head shaking, wings removed from the body to breath simpler) and intermittent severe diarrhea (green watery droppings).

Non-infected controls showed no macroscopic lesions. Macroscopic lesions were most severe for group 3 and total mean scores per tissue over all dpi were always higher than for groups 1 and 2, except for the jejunum. Mean lesion scores for groups 1 and 2 were comparable, although the total mean scores per tissue over all dpi were the highest for group 2, with the exception of the trachea, the lung and the spleen (Table 4).

3.2.3. *C. psittaci* excretion

Non-infected controls shed no *C. psittaci*. Pharyngeal excretion in all 3 infected groups was the same at 2 dpi (Table 5). Cloacal excretion was not observed at that time. Pharyngeal excretion augmented in all infected groups, but especially for group 3, followed by group 2 and 1, respectively. Pharyngeal excretion for group 3 reached a maximum score of 6, while the maximum pharyngeal excretion score for groups 1 and 2 was 5. Cloacal excretion was observed in all infected groups from 4 dpi onwards and at that time scores were the same for all infected groups. Cloacal excretion gradually increased during the experiment and was the highest for group 3 (max score of 5) followed by groups 2 and 1 (both max score of 4), respectively. At 34 dpi, all animals left in groups 1 and 2, were still excreting *C. psittaci* in their

faeces. The last remaining chicken of group 3, being euthanized at 21 dpi, was also excreting *C. psittaci*.

Table 4. Mean scores for the macroscopic lesions in euthanized or deceased chickens infected with the genotype B strain 10/423, genotype B strain 10/525 or genotype D strain 10/298 till 34 dpi.

	dpi	N[a]	Conj	Conc	Sinus	Trach	Lung	T.airs	A.airs	Peric	Liver	Spleen	Kidn	Jejun	O/T
Group 1	2	2+0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2+0	2	1	0	0	1	1	1	0	0	0	0	0	0
B-10/423)	6	2+0	1	1	0	0	1	1	1	0	0	0	0	0	0
	8	2+0	1	1	0	1	2	2	2	1	1	1	1	0	0
	10	2+0	2	2	1	1	3	3	3	2	1	1	1	1	0
	14	2+0	1	2	1	2	3	3	3	2	1	1	1	1	0
	17	2+0	1	1	1	1	3	2	2	1	1	1	1	1	0
	21	2+0	1	1	1	0	2	1	1	0	0	1	0	1	0
	24	2+0	1	0	0	0	2	1	1	0	0	2	0	1	0
	28	2+0	1	0	0	0	2	1	1	0	0	2	0	1	0
	34	2+0	1	0	0	0	2	1	1	0	0	2	0	1	0
	T[b]	22+0	12	9	4	5	21	16	16	6	4	11	4	7	0
Group 2	2	2+0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2+0	1	1	0	0	1	1	1	0	0	0	0	0	0
B-10/525)	6	2+0	2	1	0	0	1	1	1	0	0	0	0	1	0
	8	2+0	2	2	1	1	2	2	3	1	1	1	1	1	0
	10	2+0	2	2	1	1	3	3	3	2	2	2	1	1	0
	14	2+0	1	2	1	1	3	3	3	2	1	2	1	1	0
	17	2+0	1	1	1	1	2	3	3	2	1	1	1	1	0
	21	2+0	1	1	1	0	2	3	3	1	0	1	1	1	0
	24	2+0	1	0	0	0	2	1	1	0	0	1	1	1	0
	28	2+0	1	0	0	0	2	1	1	0	0	0	0	1	0
	34	2+0	1	0	0	0	2	1	1	0	0	0	0	1	0
	T[b]	22+0	13	10	5	4	20	19	20	8	5	8	6	9	0
Group 3	2	2+0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2+0	3	1	1	2	1	2	2	0	0	1	0	0	0
D-10/298)	6	1+1	3	1	1	2	3	3	3	2	0	1	3	0	0
	7	0+3	3	2	2	2	3	3	3	3	3	3	3	1	0
	8	0+5	2	2	2	2	3	3	3	2	3	3	3	1	0
	9	0+1	2	2	2	2	3	3	3	2	3	3	2	1	1
	10	0+2	2	2	2	3	3	3	3	3	2	3	3	1	1
	14	2+0	1	2	1	2	3	3	3	3	2	3	2	1	1
	17	2+0	2	2	2	1	2	3	3	2	2	3	1	1	1
	21	1+0	1	2	2	0	1	2	2	1	1	2	1	1	1
	T[b]	10+12	19	16	15	16	22	25	25	18	16	22	18	7	6

dpi: days post infection; Conj: conjunctiva; Conc: conchae; Trach: trachea; T. Airs: thoracic air sac; A. airs: abdominal air sac; Peric: pericardium; Kidn: kidney; Jejun: jejunum; O/T: ovary/testes

[a] Number of chickens (euthanized + deceased)

[b] Total score over all dpi.

Table 5. Culture scores for pharyngeal and cloacal *C. psittaci* excretion in euthanized (n) ordeceased chickens (n*).

dpi	Group 1 (10/423)		Group 2 (10/525)		Group 3 (10/298)	
	Pharyngeal	**Cloacal**	**Pharyngeal**	**Cloacal**	**Pharyngeal**	**Cloacal**
2	1 (2)	0 (2)	1 (2)	0 (2)	1 (2)	0 (2)
4	2 (2)	1 (2)	4 (2)	1 (2)	3 (1)/5 (1)	1 (2)
6	5 (2)	1 (2)	4 (2)	3 (2)	5 (1)/6 (1*)	3 (1)/5 (1*)
7	NA	NA	NA	NA	5 (3*)	5 (3*)
8	5 (2)	2 (2)	5 (2)	4 (2)	5 (5*)	5 (5*)
9	NA	NA	NA	NA	5 (1*)	5 (1*)
10	4 (1)/5 (1)	3 (1)/4 (1)	4 (2)	3 (1)/4 (1)	5 (2*)	5 (2*)
14	4 (1)/5 (1)	1 (1)/2 (1)	4 (1)/5 (1)	1 (1)/2 (1)	6 (2)	4 (2)
17	1 (2)	3 (2)	3 (2)	1 (2)	4 (1)/5 (1)	4 (2)
21	2 (2)	3 (2)	3 (2)	4 (2)	2 (1)	2 (1)
24	1 (2)	1 (1)/2 (1)	3 (1) / 5 (1)	1 (2)	-	-
28	1 (2)	1 (2)	3 (2)	4 (2)	-	-
34	0 (1)/1 (1)	1 (2)	1 (2)	1 (1)/2 (1)	-	-
Total score	55 (22)	37 (22)	73 (22)	49 (22)	99 (22)	83 (22)

dpi: days post infection; NA: not applicable as no euthanasia was planned and no chickens deceased.

3.2.4. *C. psittaci* replication in tissues and examination of the lungs for contaminants

Lungs of animals examined at 4 and 8 dpi contained no contaminating bacteria or viruses. *C. psittaci* was absent in tissues of non-infected controls. *Chlamydia* was present in all infected birds with the exception of birds of groups 1 and 2, euthanized at 2 dpi (Table 6). At that time, *C. psittaci* could already be detected (albeit low scores) in the upper (conchae) and lower respiratory tract (lung and thoracal air sac) of 1 of 2 euthanized birds of group 3.

Euthanized chickens of groups 1 and 2, both infected with genotype B, became positive at 4 dpi. The upper respiratory tract of birds of both groups remained positive till 17 dpi and scores were albeit comparable as they ranged between 0.5 and 1. For group 2, scores for tissues of the lower respiratory tract increased more rapidly than for group 1 and they remained high till 21 dpi, while high scores for group 1 were only noticed till 14 dpi. The pericardium in both groups became positive at 8 dpi and remained positive for 17 and 21 dpi for group 1 and 2, respectively. Scores were identical. At 8 dpi, a systemic infection was observed in both groups as liver, spleen and kidneys became positive, which lasted till the end of the experiment at 34 dpi.

Maximum scores for these tissues were higher for group 2 than for group 1. The jejunum in both groups remained positive till 34 dpi and scores were the same for both groups.

Table 6. Mean scores for the presence of *C. psittaci* in tissues of euthanized or deceased chickens infected with the genotype B strain 10/423, genotype B strain 10/525 or genotype D strain 10/298

	dpi	N[a]	Conj	Conc	Sinus	Trach	Lung	T.airs	A.airs	Peric	Liver	Spleen	Kidn	Jejun	O/T
Group 1	2	2+0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2+0	0.5	0.5	0	0	0.5	1	1	0	0	0	0	0	0
B-10/423)	6	2+0	0.5	0.5	0	0.5	1	1	1	0	0	0	0	0	0
	8	2+0	1	0.5	0.5	0.5	2	2	2	1.5	0.5	0.5	0.5	0	0
	10	2+0	1	1	0.5	0.5	3	4	4	3	0.5	1.5	0.5	1	0
	14	2+0	0.5	1	1	1	1.5	3.5	3.5	3	0.5	0	0.5	1	0
	17	2+0	0	0	1	1	1	2	2	2	0.5	1	0.5	1	0
	21	2+0	0	0	0	0	1	0.5	1	0	0	0.5	0	0.5	0
	24	2+0	0	0	0	0	1	1	1	0	0	1	0	0.5	0
	28	2+0	0	0	0	0	1	0	1	0	0	1	0	0.5	0
	34	2+0	0	0	0	0	1	0	1	0	0	2	0	0.5	0
	T[b]	22+0	3.5	3.5	3	3.5	13	15	17.5	9.5	2	7.5	2	5	0
Group 2	2	2+0	0	0	0	0	0	0	0	0	0	0	0	0	0
(genotype	4	2+0	0.5	0.5	0	0	0.5	1	1	0	0	0	0	0	0
B-10/525)	6	2+0	0.5	0.5	0	0.5	1.5	1	1	0	0	0	0	0.5	0
	8	2+0	1	1	0.5	1	2.5	2	3	1.5	0.5	2	0.5	1	0
	10	2+0	1	1	1	1	2	2.5	4	3	1	3	1	1	0
	14	2+0	0.5	1	1	1	3	3	3	3	1	2	1	1	0
	17	2+0	1	1	1	1	3	3	3	2	0.5	1	0.5	1	0
	21	2+0	0	0	0	0	2	3	3	1	0	1	0.5	0.5	0
	24	2+0	0	0	0	0	0.5	1	1	0	0	2	0	0.5	0
	28	2+0	0	0	0	0	1	1.5	1.5	0	0	0	0	0.5	0
	34	2+0	0	0	0	0	1	1.5	1.5	0	0	0	0	0.5	0
	T[b]	22+0	4.5	5	3.5	4.5	17	19.5	22	10.5	3	11	3.5	6.5	0
Group 3	2	2+0	0	0.5	0	0	0.5	0.5	0	0	0	0	0	0	0
(genotype	4	2+0	0.5	2	1	2	1	2	2	1	0	0	0	0	0
D-10/298)	6	1+1	1	3	1	2	4	5	5	3	1	1	3	1	0
	7	0+3	1	2	2	2	2	5	5	3	2	2	3	1	1
	8	0+5	1.5	2	2	2	2	5	5	2	1	4	1	1	1
	9	0+1	1.5	2	2	2	3	5	5	2	1	3	0	1	1
	10	0+2	1	2	2	3	3	5	5	2	1	3	2	1	0.5
	14	2+0	0.5	1	1	2	3	5	5	2	1	3	2	1	1
	17	2+0	0	0	1	1	3.5	3	4	1	1	2	2	1	0
	21	1+0	1	0	2	0	3	5	5	1	1	4	1	1	0
	T[b]	10+12	8	14.5	14	16	25	40.5	41	17	10	22	14	8	4.5

dpi: days post infection; Conj: conjunctiva; Conc: conchae; Trach: trachea; T. Airs: thoracic air sac; A. airs: abdominal air sac; Peric: pericardium; Kidn: kidney; Jejun: jejunum; O/T: ovary/testes

[a] Number of chickens (euthanized + deceased)

[b] Total score over all dpi.

For group 3, the conjunctiva and the upper respiratory tract were still positive at 21 dpi, which was not the case for groups 1 and 2. Replication was more intense as the maximum score

noticed for the upper respiratory tract was 3 (conchae and trachea), while it was only 1 for groups 1 and 2. The same was true for the lower respiratory tract, with a very intense *Chlamydia* replication between 6 and 21 dpi. Replication in the pericardium also lasted longer (from 4 till 21 dpi), but scores were comparable to the ones noticed for groups 1 and 2. At 6 dpi, a systemic infection was observed as liver, spleen, kidney and jejenum became positive. Score for the liver, kidney and especially the spleen were higher than the ones observed in groups 1 and 2. Remarkably, *C. psittaci* was also discovered in the testes and ovaria, which was not the case in groups 1 and 2.

3.2.5. Histopathology

Microscopic lesions were absent in the lungs, thoracic air sac and spleen of the control chickens. Histopathological findings were present in all infected groups and are summarized in Table 7. Bronchitis was characterized by: i) formation of follicles in the bronchus associated lymphoid tissue (BALT), ii) lymphocytic infiltrates in the bronchial wall, sometimes with some heterophils, and iii) serofibrinous exudate in the bronchial lumen associated with heterophils. Bronchitis was most severe in group 3 at day 4 dpi and still moderately present at 21 dpi. Inflammation was more severe in group 2 compared to group 1, but was absent from 24 dpi in group 2, whilst still slightly present at 34 dpi in group 1.

Pneumonitis was characterized by: i) lymphocytic infiltrates in the lung tissue, ii) serofibrinous exudate associated with heterophils in the lumen of the tertiary bronchus and or atria/infundibula, iii) epithelial hyperplasia of atria/infundibula. Pneumonitis was also most severe in group 3 at 4 and 6 dpi. Later on, pneumonitis was more severe in groups 1 and 2, in which inflammation was still present at 34 dpi. Pleuritis was present in all infected groups and was characterized by: i) lymphocytic infiltrates, ii) serofibrinous exudation and iii) mesothelial hyperplasia with proliferation of the serosal lining. Pleuritis was most severe in group 1, in which inflammation was still present at 34 dpi. In contrast, inflammation was absent at 14 and 34 dpi in groups 3 and 2, respectively.

Table 7. Histopathological lesions in *C. psittaci* infected chickens

Lesion[a]	Average score for histopathological lesions in 2 chickens of group																					
	Group 1 (10/428) on dpi								Group 2 (10/525) on dpi								Group 3 (10/298) on dpi					
	4	6	10	14	21	24	34	**Total**	4	6	10	14	21	24	34	**Total**	4	6[b]	10	14	21[b]	**Total**
Bronchitis	0	0	2	1	1.5	1.5	0.5	**6.5**	0	0.5	3	3	2	0	0	**8.5**	3.5	3	NT	1.5	3	**11**
Pneumonitis	0	1	3	3	2	2.5	3	**14.5**	0	1	1.5	3	3	1.5	1	**11**	3.5	3	NT	0	1	**7.5**
Pleuritis	0	0	1.5	1	1.5	1.5	0.5	**6**	0	0	1.5	1.5	1	1	0	**5**	0.5	1	NT	0	0	**1.5**
Aerosacculitis	0	1	3.5	4.5	3	3	2	**17**	0	1	3.5	3.5	4.5	3	2	**17.5**	3.5	3	NT	3.5	0	**10**
splenitis	0	0	4	4	4	4	4.5	**20.5**	0	0	4	4	4.5	4	4	**20.5**	4.5	4	NT	3.5	4	**16**
Serositis	0	0	1	1	0	1	0	**3**	0	0	0	0	0	0	0	**0**	0	0	NT	0	0	**0**

[a] Histological changes: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked and 5 = severe

[b] only 1 instead of 2 chickens examined, as one animal was already dead at 6 dpi and only one animal was left at 21 dpi.

NT not tested.

Aerosacculitis was characterized by: i) lymphocytic infiltrate, sometimes associated with follicle formation, ii) serofibrinous exudation, iii) mesothelial hyperplasia, iv) mesothelial necrosis with formation of fibrino-necrotic membranes on the surface and v) proliferation of fibroblastic tissue as a repair reaction after exudation and/or necrosis. Aerosacculitis was more severe in groups 1 and 2 compared to group 3, especially at 14 dpi (Figure 1) in group 1 and 21 dpi in group 2. For group 3, aerosacculitis was prominent in all chickens examined before 21 dpi.

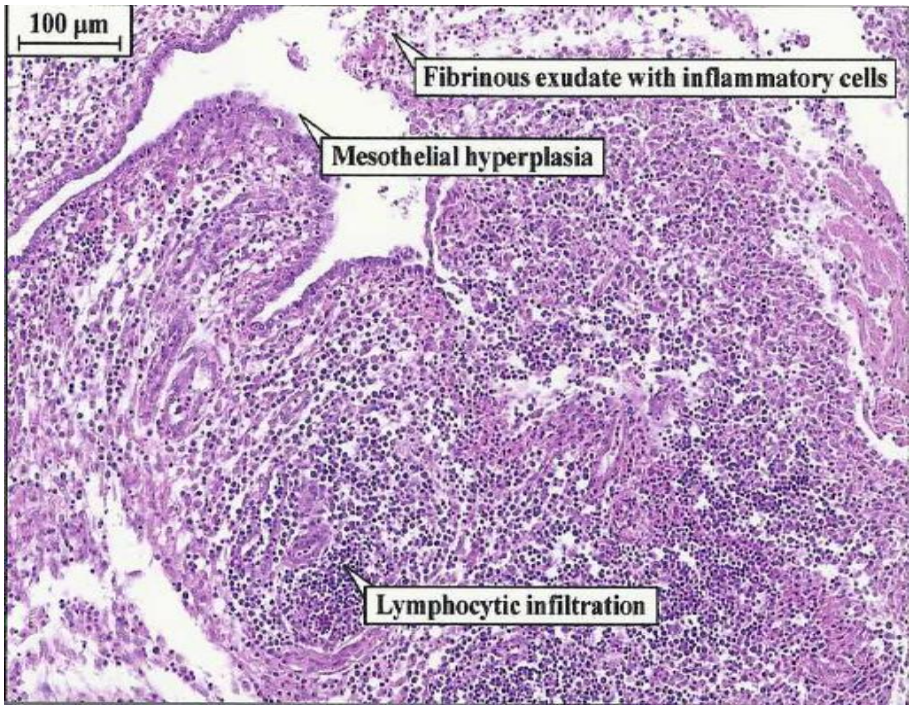

Figure 1. Haematoxylin and eosin staining of the thoracic air sac of a chicken infected with strain 10/423 at 14 dpi. Note the infiltration of lymphocytes, the presence of fibrinous exudate with inflammatory cells and the mesothelial hyperplasia (20X).

Splenitis was characterized by: i) hyperplasia of the reticulo-endothelial system with proliferation of the macrophages present in the spleen, signifying aspecific stimulation of the immune system, ii) hyperplasia of the lymphoid system with proliferation of lymphoid cells characteristic for specific stimulation of the immune system. Splenitis was moderate to severe in all 3 infected groups from 10 dpi onwards in groups 1 and 2 and from 4 dpi in group 3. Additionally, in group 1, a minimal to slight inflammation of the splenic capsule or serosa was noted until 24 dpi. Serositis of the splenic capsule was characterized by lymphocytic infiltration of the serosal surface with exudation and fibroblastic proliferation.

4. Discussion

Limited epidemiological data on *C. psittaci* infections in chickens from 1960 to 2000 indicate that chickens are relatively resistant to disease. Acute infection progressing to disease and mortality was believed to occur only in young birds, and the incidence of actual epidemics was very low (Storz *et al*., 1963; Barr *et al*., 1986; Bracewell and Bevan, 1986; Schmeer *et al*., 1986; Malkinson *et al*., 1987; Arzey and Arzey, 1990; Wittenbrink *et al*., 193; Hafez *et al*., 1994). However, diagnosis at that time was suboptimal and mainly focused on outbreaks linked to increased mortality, as occurred in those days in commercially raised turkeys, ducks and pigeons (Page and Grimes, 1984).

However, natural infections in chickens are still believed to be unapparent and transient although *C. psittaci* strains isolated from turkeys did cause similar pathology and mortality in chickens as in turkeys (Suwa *et al.*, 1990). But, in fact, we only have limited information on the occurrence of *C. psittaci* in chickens. Etiological diagnosis of respiratory disease in poultry is mostly only performed if the initiated antibiotic treatment fails. Although antibiotic usage decreased the last years, antibiotics are still frequently used and among them are the ones being active against *C. psittaci*. A recent report on veterinary antibiotic consumption in Belgium (BelVet-SAC report 2012; www.belvetsac.ugent.be) concluded that the three most applied antimicrobial classes are: 1) sulphonamides and trimethoprim, followed by 2) tetracyclines (most effective against *C. psittaci*) and 3) penicillins, which induce persistent *Chlamydia* infections (Goellner *et al*., 2006). In France, tetracylines are most frequently used (Moulin *et al*., 2008). Thus, respiratory disease due to *C. psittaci* might often be solved without the need for a proper diagnosis.

Although a pathogen of humans, little is known on the current epidemiology and pathology of *C. psittaci* in chickens. One of the reasons for our ignorance is that *C. psittaci* is not included in routine diagnosis as culture requires biosafety level 3 and the sensitivity and/or specificity of commercial antigen or antibody detection kits is not as it should be (Vanrompay *et al*., 1994; Sting *et al*., 2006; Verminnen *et al*., 2006; Geigenfeind and Haag-Wackernagel, 2010). More

recently, nucleic acid amplification techniques (NAAT's) have given us the opportunity to detect *C. psittaci* in a fast, sensitive and specific way (Sachse *et al*., 2009). Moreover NAAT's allowed molecular characterization of *C. psittaci* and rapid tracing of zoonotic sources (Branley *et al*., 2008) and made it possible to detect a new chlamydial agent in French and Australian commercially raised chickens (Laroucau *et al*., 2009; Robertson *et al*., 2010).

Ever since applying NAAT's, *C. psittaci* has been detected more often in chickens. Virulent *C. psittaci* strains were detected by NAAT's and isolated from diseased chickens raised in Australia, France, China and Germany (Yang *et al*., 2007; Gaede *et al*., 2008; Zhang *et al*., 2008; Laroucau *et al*., 2009; Robertson *et al*., 2010; Zhou *et al*., 2010). Recently, *C. psittaci* was detected in a Belgian chicken slaughterhouse and also in a Belgian chicken hatchery. Zoonotic transmission occurred (Dickx *et al*., 2010; Dickx *et al*., 2011).

The present study demonstrates the occurrence of highly and less virulent *C. psittaci* strains in broilers raised in Belgium and Northern France. Therefore, the statement that *C. psittaci* infections occurred less frequently in chickens has to be reconsidered, as our data suggest that *C. psittaci* is (re)-emerging in chickens. Diagnosis has been improved, but *C. psittaci* strains might also have adapted for replication and survival in chickens. Moreover, as our results clearly demonstrate the high prevalence of *C. psittaci* genotype B and D strains, the finding of *C. psittaci* might not be regarded as a curiosity.

Surprisingly, we are the first to study the pathology of chicken-derived *C. psittaci* strains in chickens. We could only find 5 reports on experimental infections in chickens, but these either used strains isolated from: i) a budgerigar (Izawa-1; genotype A), a parrot (GCP-1) or a pigeon (P-1041) (Takahashi *et al*., 1988a, 1988b), ii) a turkey (strain C-1, Bankowski *et al*., 1967, 1968; no strain or genotype specified, Suwa *et al*., 1990), or iii) ruminants (B-577, Bo-Yokohama, SPV-789) (Takahashi *et al*., 1988b). Also, they did not use the natural route of infection, namely inhalation of aerosols. *Chlamydiae* were directly injected into the air sac or trachea, or chickens were infected orally. The avian strains (10^5 ELD_{50}) used by Takahashi *et al.* (1988b), induced a generalized infection within 10 dpi followed by death in 8-day-old White Leghorn chickens. Strains isolated from *Psittacidae* were more virulent than the one pigeon

strain used, as they caused higher mortality in chickens. Strains derived from ruminants were far less pathogenic to chickens than avian strains.

The presently obtained strains from Belgian and French broilers were *C. psittaci* genotype B or D and created disease in experimentally infected SPF chickens. Thus, *C. psittaci* is a primary pathogen for chickens. Bacterial isolation attempts and PCR for mycoplasma species, aMPV, IB and ILTV indicated that no contaminating microorganisms contributed to the high mortality in group 3 and the pathology observed in all infected SPF chickens. Differences in pathology were observed: genotype D was more virulent than genotype B. The same has been observed for SPF turkeys (Vanrompay *et al*., 1994, 1995).

When *C. psittaci* infects chickens, it is often considered to co-infect with a virus, another bacterium or even fungi, although only three such case reports have been described (Malkinson *et al*., 1987; Reetz and Schulze, 1995; Shi *et al*., 2003). However, Beeckman *et al*., (2010), determined the cytokine responses following *C. psittaci* infection of chicken macrophages. High IL-10 and no TGF-β4 responses were observed. This could induce macrophage deactivation and NF-κB suppression and thereby, could dampen innate immunity, rendering the birds more susceptible to other pathogens.

In conclusion, *C. psittaci* infections are apparently emerging in chickens. We could prove Hill-Evans postulates for chicken-derived *C. psittaci* genotype B and D strains. Chicken-processing plant employees should be considered a risk group for human psittacosis. There is a need for higher awareness and for efficient risk assessment and management.

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Part C

Chlamydia psittaci strains from broiler chickens induce histopathological lesions and mortality in specific-pathogen-free chickens

Adapted from:

Lizi Yin[#], Isabelle Kalmar[#], Koen Chiers, Isolde Debyse and D. Vanrompay. *Chlamydia psittaci* strains from broiler chickens induce histopathological lesions and mortality in specific-pathogen-free chickens. Avian Pathology. (submitted)

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Abstract

Chlamydia psittaci is an obligate intracellular, Gram-negative bacterium causing respiratory disease in poultry. *Chlamydia* infections in chickens are globally emerging. However, data on pathology of *C. psittaci* in chickens are still scarce. We perform a detailed study on the histopathological lesions induced by two *C. psittaci* outer membrane protein A (*ompA*) genotype B strains (10/423 and 10/525) and one genotype D strain (10/298) in experimentally infected (aerosol) SPF chickens. The strains were derived from Belgian and French commercially raised broilers with pneumonia. Both genotype B and D strains induced conjunctivitis, rhinitis, sinusitis, tracheitis, bronchitis, pneumonitis, airsacculitis, splenitis, hepatitis, nephritis and enteritis in sequentially (days 2 to 34 post infection) euthanized chickens. Inflammation of the ovaries was only observed in genotype D infected chickens. Overall, the genotype D stain caused more severe histopathological lesions as illustrated by higher mean lesion scores per tissue examined and mortality (54.5%) early upon infection. The genotype D strain seemed to replicate faster as severity of the lesions increased more quickly. *C. psittaci* is a primary pathogen in chickens and efficient monitoring and control of this emerging zoonotic pathogen is urgently needed.

Keywords: *Chlamydia psittaci*, chicken, broilers, histopathology, respiratory disease

1. Introduction

Chlamydia psittaci is an obligate intracellular, Gram-negative bacterium causing respiratory disease in poultry and pet birds. *Chlamydia* infections in chickens are globally emerging, as demonstrated by epidemiological studies and case reports published throughout the world during the past five years (Yang *et al*., 2007; Gaede *et al*., 2008; Laroucau *et al*., 2009; Zhang *et al*., 2008; Dickx *et al*., 2010, 2011; Robertson *et al*., 2010; Zhou *et al*., 2010; Yin *et al*., 2013; Zocevic *et al*., 2012).

However, data on pathology of *C. psittaci* in chickens are still scarce. We could only find 6 reports on experimental infections in chickens. Strains from the following birds and mammals have been used in these studies: 1) a budgerigar (Izawa-1; genotype A), 2) a parrot (GCP-1; no genotype specified), 3) a pigeon (P-1041; no genotype specified) (Takahashi *et al*., 1988a, 1988b), 4) turkeys (strain C-1; no strain or genotype specified and the Turkey/California/181 strain; no genotype specified) (Bankowski *et al*., 1967, 1968; Suwa *et al*., 1990; Suwa and Itakura, 1992), and 5) ruminants (B-577, Bo-Yokohama and SPV-789) (Takahashi *et al*., 1988a). The avian strains (10^5 Egg Lethal Dose 50) used by Takahashi *et al.* (1988a), induced a generalized infection within 10 dpi followed by death in White Leghorn chickens. Psittacine strains were more virulent than the pigeon strain, as they caused higher mortality. Ruminant strains were far less pathogenic to chickens than avian strains.

Thus, chicken-derived strains were not used and only one of the strains was genotyped. *C. psittaci* is currently classified into the well-characterized outer membrane protein A (*ompA*) genotypes A-F and E/B. Moreover, chickens were not infected by the natural route, namely by inhalation of aerosols. Instead, *Chlamydiae* were directly injected into the air sac or trachea, or chickens were infected orally. Only Suwa *et al*., (1990, 1992) used SPF chickens.

So far genotypes B, C, D, F and E/B have been found in chickens (Gaede *et al*., 2008; Zhang *et al*., 2008; Dickx *et al*., 2010, 2011; Zhou *et al*., 2010; Yin *et al*., 2013). Genotypes B and D have been found most often but less is known on their pathology in chickens. The purpose of the present study was to perform a detailed study of the histopathological lesions induced by

chicken-derived *C. psittaci* genotype B and D strains in experimentally infected (aerosol) SPF chickens. It would allow us to examine the general assumption that *C. psittaci* is not or less pathogenic to chickens and therefore is of no importance in industrial raised chickens.

2. Materials and Methods

2.1. *C. psittaci.*

The following strains were used: i) *C. psittaci* genotype B strain 10/423 (isolated from a Belgian farm, from a broiler with pneumonia), ii) *C. psittaci* genotype B strain 10/525 (originated from another Belgian broiler farm, also associated with pneumonia), and iii) *C. psittaci* genotype D strain 10/298 (originated from a French farm, from a broiler with pneumonia) (Yin *et al*., 2013). Molecular characterization has been performed by a genotype-specific real-time PCR, allowing the identification of the outer membrane protein A (*ompA*) genotypes A to F and E/B (Geens *et al*., 2005a) and by Multi Locus Sequence Typing (MLST) using seven housekeeping genes (*enoA, fumC, gatA, gidA, hemN, hlfX, oppA*) (Pannekoek *et al*., 2010). Strains were grown in Buffalo Green Monkey (BGM) cells, as previously described (Vanrompay *et al*., 1992). Bacterial titration was performed by the method of Spearman and Kaerber (Mayr *et al*., 1974) to determine the 50% tissue culture infective dose ($TCID_{50}$) per ml.

2.2. Experimental infection of SPF chickens.

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University (EC 2010/054). Briefly, 4 groups of 22 day-old SPF chickens (Lohman, Cuxhaven, Germany) were individually tagged and housed in separate negative pressure isolators (IM1500, Montair, Sevenum, The Netherlands). At the age one week, groups 1-3 were exposed for 1 h to an aerosol of 10^6 $TCID_{50}$ *C. psittaci* suspended in PBS (5 µm droplets; Cirrus™ nebulizer; Lameris, Aartselaar, Belgium). A fourth group received an aerosol of PBS and served as non-infected control. Groups 1, 2 and 3 were infected with *C. psittaci* genotype B strain 10/423, *C. psittaci* genotype B strain 10/525 and with *C. psittaci* genotype D strain 10/298, respectively.

2.3. Sampling, *C. psittaci* detection and histopathology.

At 2, 4, 6, 8, 10, 14, 17, 21, 24, 28 and 34 dpi, two chickens from all groups were euthanized. The presence of *C. psittaci* was confirmed by examination (600X, Nikon Eclipse TE2000-E, Japan) of 5 µm thick frozen tissue sections (CM1950 cryotome; Leica Microsystems, Belgium) of the lungs, the liver and the spleen using the IMAGEN^{TM}*Chlamydia* immunofluorescence staining (Oxoid, United Kingdom). Tissue samples of the conjunctiva, conchae, sinus, trachea, lungs, abdominal and thoracic air sacs, pericardium, spleen, liver, kidney, jejunum and ovary/testis were taken for histopathology. They were fixed in 10% phosphate-buffered formalin, processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. All slides were examined microscopically (Leitz, New York, USA). Microscopic findings were graded [minimal histological change (1), slight (2), moderate (3), marked (4) or severe (5)].

3. Results and Discussion

Immunofluorescence staining of frozen tissue sections confirmed the presence of *C. psittaci* in all infected animals, while *C. psittaci* was absent in control tissues. The presence of *C. psittaci* was in accordance with the severity of the histopathological lesions. Lesions were especially pronounced in the respiratory tract resulting in a systemic infection in all infected chickens. Eleven of 22 (50%) animals of group 3 died between 7 and 10 days post infection (dpi), while all chickens of groups 1 and 2 survived the infection.

The mean lesion scores are presented in Table 1. The maximum mean lesion scores (MMLS) for the conchae, sinus, trachea, air sacs, spleen and jejunum were identical for groups 1 to 3. However, lesions appeared quicker in animals of group 3 (4 dpi) and conjunctivitis, bronchitis, pneumonitis, pericarditis, hepatitis and nephritis were most severe in chickens of group 3. Mortality (54.5%), only observed for group 3, at days 6(n = 1), 7 (n = 3), 8(n= 5), 9 (n = 1) and 10 (n = 2) post infection (p.i.), was linked with the presence of severe lesions in the respiratory tract (Figure 1), the pericardium and the spleen (Figure 2) early upon infection. Results of histopathological lesions on days 6 and 14 post infection, and on the last day of the experiment (21 or 34 dpi) are discussed in

detail.

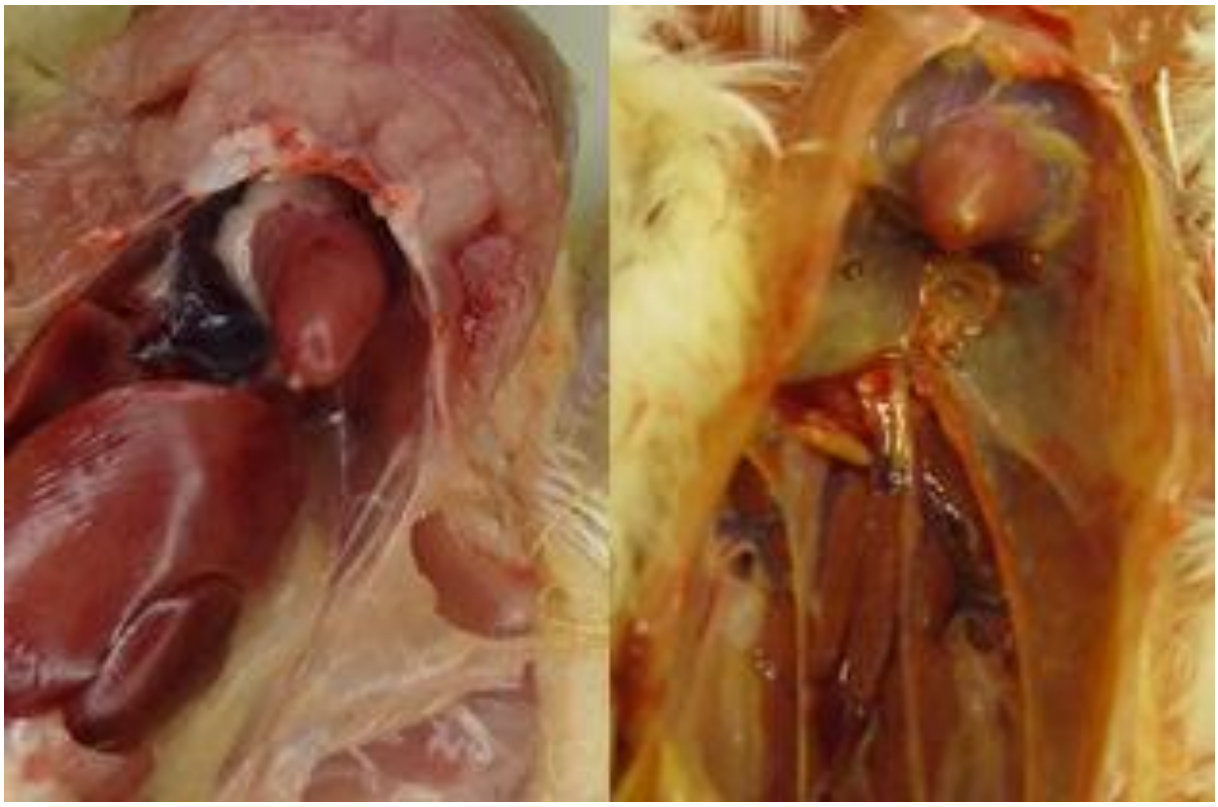

Figure 1. Diffuse fibrinous air sacculitis in a *C. psittaci* genotype D infected SPF chicken at 9 dpi (right), compared to the healthy translucent thoracic air sacs in a non-infected SPF chicken (left)

Figure 2. Enlarged and congested spleen in an SPF chicken following aerogenous infection with *C. psittaci* genotype D at 9 dpi (right), compared to the spleen of a non-infected SPF chicken (left).

3.1. Conjunctiva

Lesions in groups 1 and 2 were similar (MMLS of 1.0). At day 6 p.i. focal infiltration of lymphocytes, macrophages and heterophils in the lamina propria conjunctivae was noticed. At p.i. day 14, extensive diffuse infiltration of lymphocytes, macrophages and heterophils and

epithelial hyperplasia were observed. At p.i. day 34, infiltration of mainly lymphocytes and epithelial erosions were observed in one on two euthanized chickens of groups 1 and 2. For group 3, lesions were more severe (MMLS of 2.0), as at 6 dpi, extensive diffuse infiltration of lymphocytes, macrophages and heterophils in the lamina propria conjunctivae was noticed. At p.i. day 14 and 21, plasma cells were also present in the lamina propria conjunctivae as well as epithelial vacuolisation and epithelial erosions.

3.2. Conchae

Rhinitis, with a MMLS of 1.0 for all infected groups, was characterized by diffuse (6 dpi) or focal (14, 21 and 34 dpi) infiltration of mainly lymphocytes and some heterophils in the lamina propria.

3.3. Sinuses

Sinusitis, with a MMLS of 1.0 for all infected groups, was characterized by a mild subepithelial infiltration of lymphocytes and heterophils in the lamina propria at 6 and 14 dpi.

3.4. Trachea

Tracheitis, with a MMLS of 1.0 for all infected groups, was characterized by a moderate infiltration of heterophils at 6 (group 3) and 14 dpi (groups 1 and 2). Later on, only a moderate infiltration of lymphocytes was observed in group 3, at 21 dpi.

Table 1. Histopathological lesions in *C. psittaci* infected chickens.

Lesion	Average score for histopathological lesions in 2 chickens of group																							
	1 (10/428) on dpi									**2 (10/525) on dpi**									**3 (10/298) on dpi**					
	6[a]	8	10	14	17	21	24	28	34	6[a]	8	10	14	17	21	24	28	34	4	6[b]	8[c]	14	17	21[d]
Conjunctivitis	0.5	0.5	1	0.5	0	0	0	0	0	0.5	0.5	1	0.5	0.5	0	0	0	0	0.5	0.5	2	1	0.5	0.5
Rhinitis	0.5	0	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	0.5	0.5	0.5
Sinusitis	0	0	0.5	1	0.5	0	0	0	0	0	0	1	1	0.5	0.5	0	0	0	0.5	0.5	0.5	0.5	0.5	1
Tracheitis	0	0.5	0.5	1	0.5	0	0	0	0	0	0	1	1	0.5	0.5	0	0	0	1	1	2	1	0.5	1
Bronchitis	0	1	2	1	1	1.5	1.5	0.5	0.5	0.5	1	3	3	2	2	0	0	0	3.5	3	2.5	1.5	2.5	3
Pneumonitis	1	1.5	3	3	2.5	2	2.5	2.5	3	1	1.5	1.5	3	3	3	1.5	2.5	1	3.5	3	3	0	4.5	1
Aerosacculitis	1	1	3.5	4.5	3	3	3	2.5	2	1	2	3.5	3.5	4.5	4.5	3	3	2	3.5	3	3	3.5	4.5	0
Pericarditis	0	1	2	3	3	1	0.5	0.5	0	0	1	2	3	3	1	0.5	0.5	0	3	3.5	3.5	3.5	3.0	1
Splenitis	0	2.5	4	4	4.5	4	4	4	4.5	0	2	4	4	4	4.5	4	4	4	4.5	4	4	3.5	4.5	4
Hepatitis	0	1	1	1	0.5	0.5	0	0	0	0	1	1	1	1	1	0	0	0	1	2	2	1	1	1
Nephritis	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	2	2	1	1	1
Enteritis	0	0	0	0.5	0	0	0	0	0	0	0	0.5	0.5	0	0	0	0	0	0	0.5	0.5	0.5	0.5	0.5

[a] No lesions present on 2 and 4 dpi; [b] One chicken euthanized and one death animal examined; [c] No chickens euthanized but examination of five death animals and therefore no chickens euthanized at 10 dpi; [d] Only one animal left at 21 dpi. NT not tested.

3.5. Bronchae

Bronchitis (MMLS of 3.5 at 4 dpi) was most severe in group 3. At 6 dpi, the following were observed: i) diffuse to moderate lymphocytic infiltration in the lamina propria and in the epithelial layer of the primary, secondary and tertiary bronchus, sometimes associated with some heterophils. At p.i. day 14, lesions were characterized by: i) formation of follicles in the bronchus associated lymphoid tissue (BALT), ii) lymphocytic infiltrates in the bronchial wall, sometimes with some heterophils, and iii) serofibrinous exudate in the bronchial lumen associated with heterophils. At 21 dpi, we noticed: i) diffuse to moderate lymphocytic infiltration in the lamina propria of the primary and secondary bronchus, sometimes associated with some heterophils, and ii) multifocal, minimal infiltration of lymphocytes in the lamina propria of the tertiary bronchus associated with multifocal, minimal epithelial hyperplasia. For group 2, (MMLS of 3.0) bronchitis was characterized by slight to moderate hyperplasia of the BALT (follicular bronchiolitis) at 6 dpi. At 14 dpi, the following were noticed: i) multifocal of diffuse, moderate lymphocytic infiltration in the lamina propria of the primary, secondary and tertiary bronchus, sometimes associated with some heterophils, ii) activation of the BALT of the primary, secondary and tertiary bronchus with the formation of follicles. At 34 dpi lesions were characterized by: i) diffuse but mild lymphocytic infiltration in the lamina propria of the primary, and secondary bronchus, sometimes associated with some heterophils, and ii) multifocal, minimal lymphocytic infiltration sometimes associated with some heterophils in the lamina propria of the tertiary bronchus. For group 1 (MMLS of 2.0), no lesions were observed at 6 dpi. At 14 dpi, the following was present: i) diffuse, slight infiltration of mainly lymphocytes and some heterophils in the in the lamina propria of the primary and secondary bronchus. The same was observed for the tertiary bronchus, albeit multifocal and mild. One of the euthanized chickens showed luminal fibrinous exudate with heterophils in the bronchae, while the other one showed follicle formation in the tertiary bronchus in the absence of exudate. Similar lesions were observed on days 34 p.i., only at that time, both euthanized animals had bronchitis associated with hyperplasia of the BALT and minimal luminal serofibrinous exudate with heterophils.

3.6. Lungs

Lesions were most severe in group 3. At 6 dpi severe pneumonitis was present and lesions were characterized by: i) multifocal to diffuse, marked lymphocytic and heterophilic infiltrates in the lung tissue, and ii) serofibrinous exudate in the pleura. The following was observed at 14 dpi, when 54.5% of the chickens of group 3 already died: i) diffuse, minimal to moderate infiltration of mainly lymphocytes and some heterophils in the lung tissue, and ii) epithelial hyperplasia of atria/infundibula. At 34 dpi, formation of follicles in the BALT, minimal infiltration of lymphocytes in lung tissue and minimal hyperplasia of epithelium of lung atria/infundibula. Pneumonia in grou 1(Figure 3) and group 2 were less severe. At 6 and 14 dpi, lesions in group 2 were characterized by: i) multifocal, moderate infiltration of lymphocytes and some heterophils in the lung tissue, and ii) the formation of follicles in the BALT. Inflammation was absent in group 2 from 24 dpi onwards, but hyperplasia of the BALT was still observed till 34 dpi. At 34 dpi, inflammation was still present in group 1 and it was characterized by: i) multifocal, moderate to marked infiltration of lymphocytes and some heterophils in the lung tissue, ii) minimal to important luminal serofibrinous exudate with heterophils, iii) multifocal, minimal epithelial hyperplasia and iv) multifocal, minimal infiltration of lymphocytes and heterophils in the pleura.

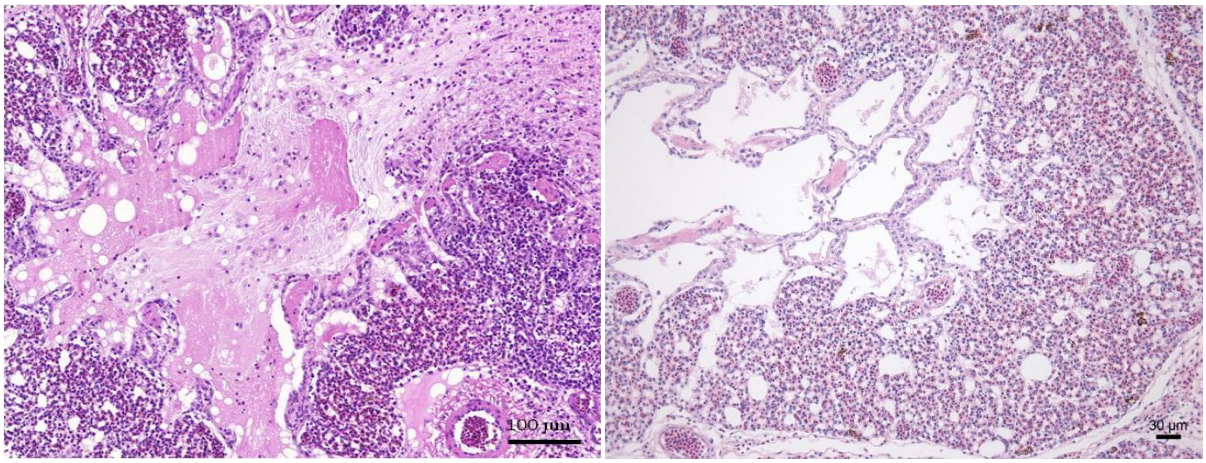

Figure 3. Lung of a chicken infected with *C. psittaci* strain 10/423 (A, 20X) and non-infected control chicken (B, 20X) at 10 days post infection. Diffuse infiltration of lymphocytes and heterophils, epithelial hyperplasia and presence of luminal fibrinous exudate with heterophils in the tertiary bronchus.

3.7. Air sacs

Histopathological changes were comparable for all groups (MMLS of 4.5) (Figure 4). However, for group 3, severe airsacculitis occurred much earlier upon infection, There were: i) lymphocytic infiltrate, sometimes associated with follicle formation, ii) serofibrinous exudation, iii) mesothelial hyperplasia, iv) mesothelial necrosis with formation of fibrino-necrotic membranes on the surface and v) proliferation of fibroblastic tissue as a repair reaction after exudation and/or necrosis.

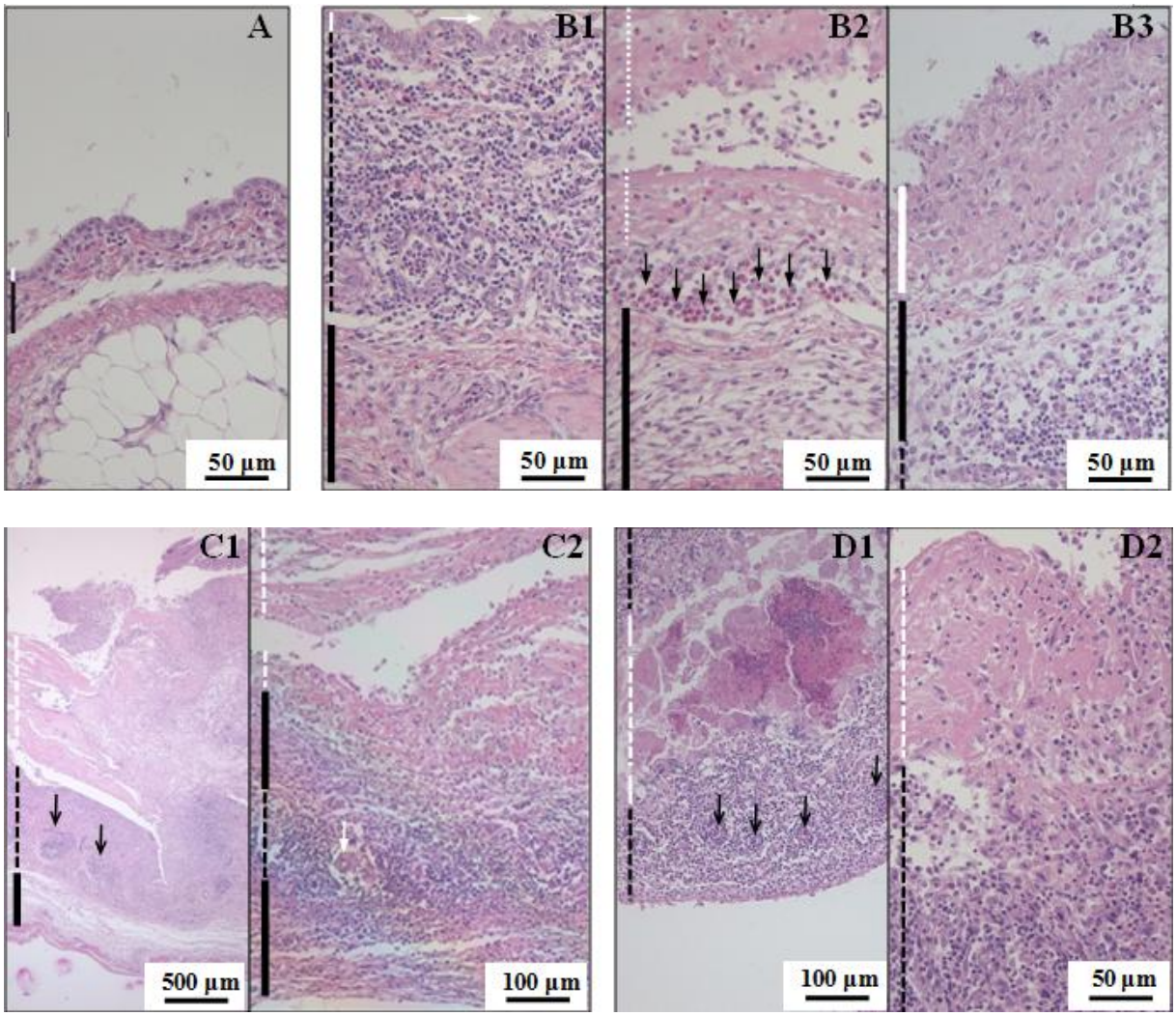

Figure 4. Hematoxylin and eosin staining of chicken thoracic air saics of an uninfected SPF chicken (photo A) and SPF chickens aerogenously infected with *C. psittaci* genotype D strain 10/298 at 4 dpi (photo B1, B2 and B3), genotype B strain 10/423 at 14 dpi (photo C1 and C2) and genotype B strain 10/525 at 14 dpi (photo D1 and D2). Foto A: normal mesothelial lining (white line) and lamina propria (black line). Fotos B to D: severe aersacculitis, which occurred much earlier following genotype D infection. Overall, histopathologic lesions were for all infection strains characterized by: mesothelial hyperplasia (thick white line) and necrosis

(white arrow) with formation of sero-fibrinous exudation (dotted white line) to fibro-necrotic membranes (dahed with line). Pathological changes in the lamina propria are lymphocytic infiltration (dashed black line) with follicle formation (black open arrows), infiltration of heterophils (black arrows) and proliferation of fibroblastic tissue (thick black line) with neovascularisation (white arrow).

3.8. Heart

Lesions were most severe in group 3 (MMLS 3.5 against 3.0 for groups 1 and 2), and occurred much earlier upon infection compared to groups 1 and 2. In group 3, severe fibrinous pericarditis was observed at p.i. days 6 and 14, while for groups 1 and 2, moderate serofibrinous pericarditis was present at day 14 p.i. Pericarditis in group 3 was characterized by: i) severe thickening of the epicardium and diffuse infiltration of lymphocytes, macrophages and heterophils in the epicardium. Thickening of the epicardium and infiltration of inflammatory cells was less pronounced in groups 1 and 2.

3.9. Spleen

Histopathological changes were severe in all groups (MMLS of 4.5) and were present until 34 dpi. However, for group 3, severe splenitis occurred much earlier upon infection. Score 4.5 meant: i) hyperplasia of the reticulo-endothelial system with proliferation of the macrophages present in the spleen, signifying aspecific stimulation of the immune system, ii) hyperplasia of the lymphoid system with proliferation of lymphoid cells characteristic for specific stimulation of the immune system, iii) marked infiltration of lymphocytes, iv) fibrin trombi (6 dpi), and v) epithelial hyperplasia. In addition, chickens of group 1 showed minimal to slight inflammation of the splenic capsule or serosa until 24 dpi.

3.10. Liver

Hepatitis was most severe in group 3, as demonstrated by the MMLS of 2.0, and was already observed at 6 dpi. At day 6, hepatitis in group 3 was characterized by: i) mild reticulosis, ii) moderate infiltration of heterophils in the space of Disse (perisinusoidal space), iii) slight proliferation of bile ducts, iv) moderate periportal infiltration of lymphocytes and macrophages. At day 6 p.i., hepatitis was absent in groups 1 and 2.

3.11. Kidneys

Histopathological changes were common for all groups but the lesions were most severe in group 3, as demonstrated by the MMLS of 2.0. Also, in group 3, nephritis was already observed at 6 dpi, when inflammation was still absent in the kidneys of the other groups. Nephritis was characterized by minimal (groups 1 and 2) to moderate (group 3) infiltration of mainly lymphocytes and heterophils throughout the parenchymatous tissue. In group 3, inflammation was observed till 21 dpi, while in groups 1 and 2, only till 14 dpi.

3.12. Jejunum

Enteritis, with a MMLS of 1.0 for all infected groups, was characterized by focal, slight infiltration of lymphocytes, macrophages, heterophils and eosinophils in the serosa. In group 3, lesions were present until 21 dpi, while only until 14 dpi for groups 1 and 2.

3.13. Ovary/testes

For group 3, at p.i. days 6 and 14, mild infiltration of lymphocytes and heterophils was observed in the ovaries. No lesions were observed in groups 1 and 2.

Overall, the observed histopathological lesions are similar as the ones described by Suwa *et al*. (1990), examining the pathology of *C. psittaci* strains derived from turkeys following an experimental infection (air sac inoculation; $10^{1.37}$ and $10^{2.37}$ ELD_{50}) of one-day-old SPF chickens and as described by Vanrompay *et al*., (1995), examining the pathology of *C. psittaci* strains isolated from a diseased pigeon (89/1326; genotype B), a diseased parakeet (84/55; genotype A) and two different turkey isolates originating from farms with respiratory disease and mortality (the Dutch strain 92/1293 and the TT-Texas Turkey strain; ATCC VR-351; both genotype D) for experimentally infected (aerosol, 10^6 $TCID_{50}$) 7-day-old SPF turkeys.

Airsacculitis seemed to be more pronounced in turkeys, as fibrinous necrotizing airsacculitis was observed in turkeys infected with the virulent strains TT or 92/1293 genotype D strains. The virulent genotype D strain 10/298, originating from chickens, induced serofibrinous airsacculitis. The latter, seems to be host-related as an infection of SPF chickens with strain 92/1292 also induces serofibrinous airsacculitis and no necrosis (Lagae *et al*., 2012,

unpublished results). Further research might focus on host-related factors, such as immune responses early upon a *C. psittaci* infection in chickens and turkeys.

C. psittaci antigen was most often demonstrated in the conjunctivae and the respiratory tract (data not presented). Thus, the liver and the spleen, which are frequently examined for diagnosis, appear to be the least desirable organs for chlamydal diagnosis. The same was noticed by Bankowski *et al*. (1968), infecting one-day-old, 35-day-old and 40-day-old non-SPF chickens, intratracheally with the C-1 strain (5×10^3 or 4×10^4 mouse-ID_{50}), originally isolated from a naturally infected Californian turkey flock.

Interestingly, the genotype D strain 10/298 caused inflammation in the ovaries and *C. psittaci* was demonstrated by immunofluorescene in the ovaries of chickens of group 3. Vertical transmission of *C. psittaci* occurs in chickens (Wittenbrink *et al*., 1993), but the extend or impact of vertical transmission on *C. psittaci* infections in chickens has never been examined.

In conclusion, *C. psittaci* is a primary pathogen for chickens and infections are currently emerging in chickens, maybe because of the reduced antibiotic use. Genotype B strains and especially the genotype D strain caused severe respiratory disease in SPF chickens with marked histopathological lesions. Our results justify a systematic *C. psittaci* examination in case of respiratory disease and/or mortality in chickens. Efficient monitoring and control of chlamydiosis in chicken flocks should be ensured, perhaps by ovotransferrine administration (Van Droogenbroeck *et al*., 2011), as it could reduce respiratory disease and *C. psittaci* zoonotic transmission (Dickx *et al*., 2010, 2011).

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Chapter Four

General discussion and perspectives

General discussion

Chlamydiaceae is a family of intracellular Gram-negative bacteria causing a variety of diseases in animals and humans. The present work is focusing on *Chlamydiaceae* infections in livestock and on the zoonotic potential of especially *C. psittaci* and *C. abortus*. **Chapter I** summarizes the current knowledge on *Chlamydiaceae* infections in livestock, focusing on infections in my homeland China. Livestock production, chlamydial diagnosis and prevention differs somewhat from the European situation, but overall it can be concluded that, as in Europe, *Chlamydiaceae* infections are an actual veterinary problem in Chinese livestock, in particular *C. abortus* infections in ruminants and *C. psittaci* infections in poultry.

In contrast to many European countries, where *C. psittaci* infections in poultry are notifiable, only enzootic abortion of ewes is notifiable in China, as it is on list C of the classification of the Ministry of Agriculture of the People's Republic of China since 2008. Sick animals are killed and the dead animals, aborted fetuses and contaminated materials (for instance placenta) are decontaminated. As in Europe, *Chlamydiaceae* are not included in a diagnostic panel of routinely examined animal pathogens.

In contrast to many European countries, *C. psittaci* and *C. abortus* infections in humans are not notifiable diseases in China's mainland, but psittacosis is a notifiable disease in Taiwan and Hong Kong. In china, awareness of clinical and economical implications of *Chlamydiaceae* in livestock is raising like in Europe and other Asian countries (Zocevic *et al*., 2012). It leads to increased biosecurity measurements, augmented financial support for vaccine research and public education (Cong *et al*., 2013).

Chapter II focuses on the occurrence of *C. abortus* infections in Belgian ruminants. Prevalence data for *C. abortus* infections in Belgian ruminant herds and flocks are not available. Therefore, we performed an epidemiological study in co-operation with the CODA-CERVA (Ukkel, Belgium). Our results showed that the prevalence of *C. abortus* among ruminants is lower than in other European countries. Lower prevalence rates are not due to vaccination efforts, as vaccination is not routinely performed in Belgium. In the UK and France, in contrast,

where *C. abortus* prevalence rates are apparently higher, vaccination of domestic ruminants is widely practiced. Our study, unlike most other reports, did however not focus on herds/flocks with fertility problems. Hence, it would be interesting to examine ruminant herds dealing with infertility and/or abortus. Also, we examined additional goat herds, as only 9 herds were included in the present study, with 2 of these being tested at large sample size (more than 10 samples per herd). Still, current results revealed *C. abortus* antibodies in one goat herd that showed a high intra-herd prevalence (52.9%, 9/17). With respect to the zoonotic risk, it could be worthwhile and perhaps wise to include goats present on children's farms in future studies. Perhaps, it is also worthwhile to perform a molecular diagnostic survey, as molecular diagnostic techniques are more sensitive and more specific than serological assays. However, the thought that antigen or gene detection has to be performed on aborted material has maybe hampered molecular diagnosis. Aborted material arriving in the laboratory is mostly in such a bad condition (post mortem decomposition), that antigen and/or gene detection is no longer possible. However, maybe we should examine fresh faecal samples or rectal swabs of ruminants, as the gut is probably the location where *C. abortus* persists.

In **Chapter III**, we examine the epidemiology and virulence of *C. psittaci* in chickens. *Chlamydia psittaci* infections and infections with atypical *Chlamydia* are apparently emerging in France and in other parts of the world (Laroucau *et al*., 2009; Zocevic *et al*., 2012, Robertson *et al*., 2010). However, until recently, it was commonly assumed that *C. psittaci* is less pathogenic for chickens than for turkeys and ducks. Perhaps this is why avian chlamydiosis is uninvestigated in chickens. Nonetheless, *C. psittaci* was isolated from symptomatic broilers and laying hens (Barr *et al*., 1986; Zhang *et al*., 2008). The isolate of Barr *et al*., (1986) originated from Australian broilers showing blindness, weight loss and coughing, whereas Zhang *et al*., (2008) reported the isolation of *C. psittaci* genotype C from Chinese laying hens with cystic oviducts. In comparative pathogenicity studies published in 1988, it was already demonstrated that chickens are susceptibility to non-chicken derived *C. psittaci* strains (Takahashi *et al*., 1988a, 1988b). Recently, a new atypical chamydial agent was isolated from asymptomatically infected French broiler chickens. This isolate was associated with a psittacosis outbreak in French poultry abattoir workers (Laroucau *et al*., 2009). These insights

led us to investigate the importance of *C. psittaci* infections in industrial raised chickens.

Chlamydia psittaci is classified into the well-characterized outer membrane protein A (*ompA*) genotypes A-F and E/B. Genotypes B, C, D, F and E/B have been found in chickens (Vanrompay *et al*., 1997; Gaede *et al*., 2008; Zhang *et al*., 2008; Dickx *et al*., 2010; Zhou *et al*., 2010) and genotypes B and D often seem to infect Belgian broilers (Vanrompay *et al*., 1997; Dickx *et al*., 2010; Dickx and Vanrompay, 2011). In **Chapter III, Part A**, *C. psittaci* genotype B (CP3) and D (92/1293) strains were used for aerogenous experimental infection of SPF chickens. Beeckman *et al*. (2010) formerly used these strains in an *in vitro* trial of chicken macrophages (HD11 cells) to study the host pathogen interactions of the low virulent *C. psittaci* genotype B reference strain CP3 (Bankowski and Page, 1959; Piraino, 1969) compared to the highly virulent *C. psittaci* genotype D strain (92/1293). Strain CP3 was isolated in 1957 from a Californian pigeon, while strain 92/1293 was isolated in 1992 from Dutch diseased turkeys (Vanrompay *et al*., 1993). In the current *in vivo* trial, we focused on studying the pathogenicity of CP3 and 92/1293 in SPF chickens in order to compare the results with the previously obtained *in vitro* (HD11 cells) data.

Lesions were present in all infected chickens and observations on the pathogenicity were in accordance with those observed during other experimental infections in chickens (Bankowski *et al*., 1967; Bankowski *et al*., 1968; Takahashi *et al*., 1988a; Takahashi *et al*., 1988b). Genotype D was more virulent than genotype B, creating mortality and more severe clinical signs and lesions. A matching difference in degree of pathology has also been observed *in vitro* while examining the developmental cycle of these strains in chicken macrophages (Beeckman *et al*., 2010). Interestingly, similar *in vivo* observations have also been made in SPF turkeys experimentally infected with strain 92/1293 or strain 89/1326. The latter is also a pigeon derived genotype B strain. As for CP3, the incubation period for the genotype B strain 89/1326 was also longer, maximal replication was delayed, the period during which bacteria were observed in the same tissue was also shorter and tissue tropism also seemed to be less extensive as compared to an infection with strain 92/1293 (Vanrompay *et al*., 1994; Vanrompay *et al*., 1995). In conclusion, non-chicken derived *C. psittaci* genotype B and D strains both created

significant lesions in experimentally infected SPF chickens, but differences in virulence and associated pathology were observed.

We could find only 6 other reports on experimental infections with *C. psittaci* in chickens. Strangely, none of these reports used an aerosol infection model. Instead, infection was performed by: 1) the intra-air-sac route (Takahashi *et al*., 1988a, 1988b; Suwa *et al*., 1990; Suwa and Itakura, 1992), 2) the peroral route (Takahashi *et al*., 1988a, 1988b), 3) the intratracheal (injection) route (Bankowski *et al*., 1967, 1968), 4) the intraperitoneal route (Bankowski *et al*., 1968), and finally 5) the intracerebral route (Bankowski *et al*., 1968). These routes involve high technical requirements for operators and are invasive to the experimental animals. In contrast, infection challenge by inhalation of an aerosol containing the inoculum is a completely non-invasive and non-painful procedure. Moreover, it most closely mimics the natural infection route of avian chlamydiosis.

In **Chapter III, Part B,** the current epidemiological status of *C. psittaci* infections in the Belgian and Northern France chicken industry was investigated by an in-house developed *C. psittaci* major outer membrane protein (MOMP) based ELISA on sera of 30 Belgian and 10 French farms. In addition, culture of pharyngeal swabs of 9 Belgian farms and organ samples of 5 Belgian and 5 French farms was performed in buffalo green monkey cells. Molecular characterization of lung isolates was done using real time PCR, *ompA* sequencing and miroarray. It was our purpose to obtain up to date epidemiological data and to obtain different *C. psittaci* strains for proving Hill-Evans postulates for chickens. All Belgian broiler, broiler breeder and layer farms and all French broiler farms were seropositive. Moreover, 96%, 93% and 90% of Belgian broilers, broiler breeders and layers were seropositive. Similarly, 91% of French broilers were seropositive. Lung tissue obtained from all five French broiler farms and 4 of 5 (80%) Belgian broiler farms were culture positive, as were pharyngeal swabs of all 9 examined Belgian broiler farms. Molecular characterization of lung isolates revealed the presence of *C. psittaci* genotype B and D in the French broilers and genotype B in the Belgian broiler farms. *Chlamydia psittaci* infections can thus be considered an emerging infectious disease in chickens. Chicken farmers and employees in chicken-processing plants should be considered a risk group for occupationally contracted psittacosis.

These results confirm the findings of Laroucau *et al*., (2009) and the more recent results of Dickx *et al*., (2011) and Deschuyffeleer *et al*., (2012), who all concluded that chlamydiosis in broiler chickens is a threat to workers in poultry processing plants. Clearly, the seroprevalence of *C. psittaci* infections in Belgian chicken farms is much higher as compared to the *C. abortus* seroprevalence in Belgian ruminants. Differences in infectivity between *C. abortus* and *C. psittaci*, in stocking densities and in farm management, as well as the predominant aerogenic spread of *C. psittaci* might explain this finding.

We proved Hill-Evans postulates by infecting SPF chickens with *C. psittaci* genotype B strains 10/423 and 10/525, and genotype D strain 10/298, which were isolated from lungs of chickens with pneumonia. Bacterial isolation attempts and PCR for mycoplasma species, aMPV, IB and ILTV indicated that no contaminating microorganisms contributed to the pathology observed in all infected SPF chickens and the high mortality noticed following *C. psittaci* genotype D infection. Thus, *C. psittaci* is a primary pathogen for chickens. This is the first experimental infection with *C. psittaci* in chickens in which infection occurred with chicken-derived isolates. The 6 above-mentioned studies used non-chicken derived strains (Bankowski *et al*., 1967, 1968; Takahashi *et al*., 1988a, 1988b; Suwa *et al*., 1990). It is impossible to compare the course of disease following experimental infections with a natural infection as systematic data on mortality, macroscopic lesions, course of bacterial excretion and histopathology in naturally infected chickens are lacking.

In **Chapter III, Part C,** the histopathological lesions induced by chicken-derived *C. psittaci* genotype B and D strains in experimentally infected (aerosol) SPF chickenswere studied in detail. This allowed us to further question the general assumption that *C. psittaci* is not or less pathogenic to chickens. The respiratory tract is described to be the most important route of *C. psittaci* infection in birds, including chickens (Barr *et al*., 1986; Malkinson *et al*., 1987; Suwa *et al*., 1990; Laroucau *et al*., 2009; Yin *et al*., 2013). The results of our study supported this opinion. Varying degrees of infiltration of lymphocytes and heterophils were observed in the respiratory tract. There wasserofibrinous exudate in the bronchae, lungs and air sacs of infected chickens. Lesions in genotype D (strain 10/298) infected chickens were significantly more

severe than the ones observed in chickens infected with the genotype B strains 10/432 or 10/525. Overall, the observed histopathological lesions were similar as described in infection trials in which chickens, turkeys or quail were infected with non-chicken derived *C. psittaci* strains. This includes Suwa *et al*. (1990), who examined the pathology of *C. psittaci* strains derived from turkeys following experimental infection by air sac inoculation ($10^{1.37}$ and $10^{2.37}$ ELD_{50}) of one-day-old SPF chickens. Vanrompay *et al*. (1995), who examined the pathology of *C. psittaci* strains isolated from a diseased pigeon (89/1326; genotype B), a diseased parakeet (84/55; genotype A) and two different turkey isolates originating from farms with respiratory disease and mortality (the Dutch strain 92/1293 and the TT-Texas Turkey strain; ATCC VR-351; both genotype D) following experimental infection (aerosol, 10^6 $TCID_{50}$) in 7-day-old SPF turkeys. And third, Batta *et al*. (1999), who examined the pathology of *C. psittaci* strains isolated from mammals in experimentally infected (intratracheally, 1 $CELD_{50}$) two-week-old Japanese quails. Airsacculitis seemed to be more pronounced in turkeys, as fibrinous necrotizing airsacculitis was observed following infection with the virulent strains TT or 92/1293 genotype D strains. The latter seems to be host-related, as an infection of SPF chickens with strain 92/1293 also induces serofibrinous airsacculitis but no necrosis. Further research might focus on host-related factors, such as immune responses early upon a *C. psittaci* infection in chickens and turkeys. Natural infected chickens often show blindness, conjunctivitis and predominant eye lesions (Barr *et al*., 1986; Malkinson *et al*., 1987; Arzey and Arzey, 1990). In our study, infiltration of lymphocytes, macrophages and heterophils, epithelial hyperplasia, epithelial erosions and epithelial vacuolisation were noticed in the lamina propria of the conjunctivae of infected chickens. Vertical transmission has been demonstrated in chickens (Wittenbrink *et al*., 1993), but its extend or impact on *C. psittaci* infections in chickens has never been examined. We found that the genotype D strain 10/298 caused inflammation in the ovaries and *C. psittaci* was demonstrated by immunofluorescence in the ovaries of these chickens.

In conclusion, *C. psittaci* infections are currently emerging in chickens, maybe because of reduced antibiotic use. However, although antibiotic usage decreased the last years, antibiotics are still frequently used and among them are the ones being active against *C. psittaci*. A recent

report on veterinary antibiotic consumption in Belgium (BelVet-SAC report 2012; www.belvetsac.ugent.be) concluded that the three most applied antimicrobial classes are: 1) sulphonamides and trimethoprim, followed by 2) tetracyclines, which is the most effective antibiotic against *C. psittaci*, and 3) penicillins, which induce persistent *Chlamydia* infections (Goellner *et al*., 2006). In France, tetracylines are most frequently used (Moulin *et al*., 2008). As far as we know, tetracycline or enrofloxacin resistant *C. psittaci* strains have not been found. In contrast, tetracycline resistant *Chlamydia suis* infections are currently emerging worldwide in swine (Schautteet *et al*., 2013).

Perspectives

Our results justify: i) a molecular epidemiological study of *C. abortus* in a larger population of Belgian ruminants, as nucleic acid amplification techniques are far more sensitive and specific than the currently available serological tests, ii) a systematic *C. psittaci* examination in case of respiratory disease and/or mortality in chickens. Efficient monitoring and control of chlamydiosis in chicken flocks should be ensured, perhaps by a vaccine or ovotransferrin administration. Ovotransferrin, which is a natural anti-microbial protein present in egg white and avian serum, has been demonstrated to reduce respiratory disease and *C. psittaci* excretion in turkeys when administered (at a dose of 5 mg per aminal) as an aerosol (Van Droogenbroeck *et al*., 2011), iii) implementation of a *C. psittaci* occupational safety & health program. Therefore, we would need the cooperation of our government and of occupational physicians.

Summary

Summary

Chlamydiaceae is a family of obligate intracellular, Gram-negative bacteria causing disease in man and animals. *C. abortus* is a major abortigenic agent in ruminants, causing ovine enzootic abortion (OEA). In addition, it is a well-recognized and potentially fatal zoonosis, presenting a major hazard to pregnant women who come in contact with livestock, particularly at lambing. In cattle, the infection is predominantly associated with genital tract disease and mastitis. *C. psittaci* causes respiratory disease (avian chlamydiosis) in birds and psittacosis or parrot-fever in man. Avian *C. psittaci* strains are classified into seven outer membrane protein A (*ompA*) genotypes: A to F and E/B. All genotypes are considered readily transmissible to man by inhalation or direct contact. Until recently, chlamydiosis associated with severe clinical disease in poultry, was thought to occur primarily in turkeys and ducks. However, current epidemiological data clearly show the widespread presence of virulent *C. psittaci* genotypes in chickens as well. So far, genotypes B, C, D, F and E/B have been found in chickens.

In **Chapter I**, an overview is given of the epidemiology, prevention, treatment and zoonotic risk of *Chlamydiaceae* infections in Chinese livestock. Differences in risk-factors, such as bio-security measures, and use of diagnostic tests and veterinary practices applied in China are compared to the West.

In **Chapter II**, we examined the occurrence of *C. abortus* in Belgian ruminants, as such prevalence data are not available. We performed a serological survey in Belgian sheep (n=988), goat (n=48) and cattle (n=1887) using the ID Screen™ *Chlamydia abortus* indirect multi-species ELISA. At sample size of 10 or more sera per herd, results revealed a seropositive herd status in 11.6% (11/95) and 14.3% (6/42) of cattle and sheep herds, respectively. In addition, 6.3% (6/95) cattle herds and 11.9% (5/42) sheep herds tested suspicious. Only 2 goat herds were tested at this sample size: one herd tested seropositive and one was suspicious. None of 38 cattle herds tested at low sample size (< 10 sera per herd) was positive and 2 herds (5.3%) were suspicious. In sheep, 6 of 280 sera from 56 herds tested at small sample size were positive, counting for an additional 6 seropositive herds in Belgium. A

high intra-herd prevalence was seen in the seropositive goat herd (52.9%; 9/17). In contrast, seroprevalence in positive cattle and sheep herds was relatively low with generally only 1 or 2 seropositive animals on 10 to 20 tested animals per herd. This may explain a higher rate of seronegativity at herd level when tested at low sample size. Seroprevalence studies in most other countries reported higher infection rates at animal level. However, most of these focus on farms with fertility problems, which could explain higher infection rates. Molecular diagnosis by the 23S rRNA-based ArrayTube™ microarray on rectal swabs sampled at 3 cattle farms (n=20 per farm) tested all negative for *Chlamydiaceae* DNA.

In **chapter III**, we focused on the epidemiology and virulence of *C. psittaci* in chickens. All our experimental infections occurred by the aerogenous route in young specific-pathogen-free (SPF) chickens. In **part A.,** we referred to the *in vitro* trial of Beeckman *et al*. (2010), who performed a study in chicken macrophages (HD11 cells) comparing host pathogen interactions of the low virulent *C. psittaci* genotype B reference strain CP3 (Bankowski and Page, 1959; Piraino, 1969) to the highly virulent *C. psittaci* genotype D strain (92/1293). Strain CP3 was isolated in 1957 from a Californian pigeon while strain 92/1293 was isolated in 1992 from Dutch diseased turkeys (Vanrompay *et al*., 1993). The genotype D strain: 1) clearly induced actin recruitment to the site of *chlamydia* entry and invaded the host cells more efficiently, 2) initiated host cell degeneration at earlier time points, and 3) survived and proliferated better in macrophages when compared to the low virulent CP3 strain. Part III. A. therefore focused on studying the pathogenicity of CP3 and 92/1293 *in vivo* in SPF chickens in order to compare the results with the previously obtained *in vitro* (HD11 cells) data. Pharyngeal and cloacal *C. psittaci* excretion was observed in all infected animals. Moreover, direct immunofluorescence staining (DIF) of tissue sections demonstrated replication in internal organs and thus systemic spread of both *C. psittaci* strains. However, infection with genotype D appeared more virulent as indicated by more severe clinical signs, namely conjunctivitis, rhinitis and dyspnoe, and histopathological lesions as compared to genotype B. In addition, in contrast to genotype B, genotype D resulted in high mortality. Results confirmed the *in vitro* data obtained by Beeckman *et al*., (2010).

In **Chapter III, part B**, the epidemiology of *C. psittaci* in commercial Belgian and French broilers was investigated. In addition, we proved Hill-Evans postulates for *C. psittaci* infections

in chickens using *C. psittaci* strains genotype B and D strains isolated from the lungs of chickens with pneumonia. In the epidemiological study, we tested sera of 30 Belgian and 10 Northern French chicken farms by use of an in-house developed *C. psittaci* major outer membrane protein (MOMP) based ELISA. Ninety-six percent, 93% and 90% of Belgian broilers, broiler breeders and layers were seropositive. Similarly, 91% of French broilers were seropositive. Next, lung tissue of broilers was sampled at a slaughterhouse and examined by culture. All five French farms and 4 of 5 (80%) examined Belgian farms were culture positive.. *C. psittaci* infections can thus be considered an emerging infectious disease in chickens. Chicken farmers and employees in chicken-processing plants should be considered a risk group for occupationally contracted psittacosis.

In **Chapter III, part C,** focuses on the histopathological lesions caused in experimentally infected SPF chickens. SPF chickens were experimentally infected with *C. psittaci* strain 10/423 (genotype B), 10/525 (genotype B) or 10/298 (genotype D), which were all isolated from Belgian or French broilers with pneumonia. All strains induced conjunctivitis, rhinitis, sinusitis, tracheitis, bronchitis, pneumonitis, airosacculitis, splenitis, hepatitis, nephritis and enteritis. Inflammation of the ovaries was only observed in genotype D infected chickens. Overall, the genotype D strain caused more severe histopathological lesions and a high mortality rate (54.5%) early upon infection. In addition, severity of lesions increased more rapidly in genotype D as compared to genotype B infected birds, which suggests faster replication in internal organs.

In conclusion, *C. psittaci* is a primary pathogen in chickens. Higher awareness for this bacterium is warranted to improve animal health as well as to diminish the occupational health risk for poultry workers. Therefore, efficient monitoring and control of this emerging zoonotic pathogen is urgently needed.

Samenvatting

De familie van de *Chlamydiaceae* vormt een groep van obligaat intracellulair Gram-negatieve bacteriën. Deze bacteriën veroorzaken infectieziekten bij mens en dier. *Chlamydia (C) abortus* is een belangrijke oorzaak van abortus bij herkauwers en veroorzaakt ovine enzootische abortus (OEA) bij schapen. *C. abortus* is ook alom gekend om zijn zoönotisch karakter. De kiem kan abortus veroorzaken bij zwangere vrouwen die in contact komen met besmette schapen en dan voornamelijk na contact met verworpen lammeren. Bij runderen wordt een *C. abortus* infectie voornamelijk geassocieerd met een infectie van de genitaaltractus en met uierontsteking. *C. psittaci* veroorzaakt ademhalingssymptomen (aviaire chlamydiosis) bij vogels en psittacosis of de papegaaienziekte bij de mens. Aviaire *C. psittaci* stammen worden ingedeeld in 7 verschillende genotypen, gaande van genotype A tot F en het genotype E/B. Deze indeling is gebaseerd op de sequentie van het 'outer membrane protein' A (*ompA*) gen. Al deze genotypen zijn potentieel overdraagbaar van vogels naar de mens. Dit gebeurt in hoofdzaak door inhalatie of direct contact. Tot voor kort werd aangenomen dat ernstige uitbraken van chlamydiosis bij pluimvee enkel voorkwamen bij kalkoenen en eenden. Recente epidemiologische data wijzen er echter op dat virulente *C. psittaci* genotypen ook wereldwijd verspreid zijn in de kippenindustrie. Bij het aanvang van dit onderzoek waren *C. psittaci* genotype B, C, D, F en E/B infecties gemeld bij kippen.

In hoofdstuk I geven we een literatuuroverzicht over de epidemiologie, preventie, behandeling en het zoönotisch risico van *Chlamydiaceae* infecties bij landbouwhuisdieren in China. Naast epidemiologische gegevens, bestuderen we hierbij de verschillen in bio-beveiliging tussen China en het Westen en de hieruit voortvloeiende risicos op infectie voor mens en dier. Tevens kijken we naar de verschillen in diagnostische methoden en behandelingen.

In hoofdstuk II onderzoeken we het voorkomen van *C. abortus* infecties bij Belgische herkauwers aangezien er voor Belgie geen epidemiologische data zijn over de prevalentie van deze zoönotische infectie. We hebben hiervoor een serologische studie uitgevoerd bij Belgische schapen (n = 988), geiten (n = 48) en runderen (n = 1887). Het serum van deze dieren werd onderzocht aan de hand van de indirecte multi-species ID Screen™ *Chlamydia abortus*

'enzyme-linked immunosorbent assay' (ELISA). Van 42 schapenbedrijven, 2 bedrijven met geiten en 95 runderbedrijven werden minimum 10 dieren per bedrijf getest. Hiervan werden 14.3% (6/42) van de schapenbedrijven, 50% (1/2) van de geitenbedrijven en 11.6% (11/95) van de runderbedrijven seropositief bevonden. In het seropositieve geitenbedrijf waren 52.9% (9/17) van de onderzochte geiten seropositief, terwijl in de positieve schapen-en runderbedrijven overwegend slechts 1 of 2 van de geteste dieren positief reageerde in de ELISA. Dit laatste verklaart dat kuddes getest aan een kleinere steekproefgrootte een lagere seropositieve status toonden. Rectale swabs van drie bijkomende runderbedrijven (n = 20 per bedrijf) werden onderzocht aan de hand van de 23S RNA ArrayTube™ microarray. Deze microarray kan gebruikt worden om het DNA van de diverse *Chlamydia* species op te sporen. De rectale swabs waren echter allemaal negatief. Over het algemeen kunnen we zeggen dat de seroprevalentie van *C. abortus* in België lager is dan in andere landen waar dergelijke studies werden uitgevoerd. Een mogelijke verklaring is dat in deze studies voornamelijk bedrijven met vruchtbaarheidsproblemen onderzocht werden.

In hoofdstuk III onderzoeken we de epidemiologie en virulentie van *C. psittaci* voor kippen. In deel A verwijzen we naar de studie uitgevoerd door Beeckman *et al*., (2010) waarbij kippenmacrofagen (HD11 cellen) gebruikt werden. Tijdens deze *in vitro* studie werd de pathogene interactie tussen een gering virulente *C. psittaci* stam (genotype B referentie stam CP3) en de HD11 gastheercel onderzocht. CP3 werd in 1957, in Californië geïsoleerd uit een duif (Bankowski and Page, 1959; Piraino, 1969). Tevens werd de pathogene interactie tussen een hoog virulente *C. psittaci* stam (genotype D stam 92/1293) en de HD11 gastheercel onderzocht. De stam 92/1293 werd in 1992 geïsoleerd uit zieke kalkoenen van een Nederlands pluimveebedrijf (Vanrompay *et al*., 1993). De genotype D stam veroorzaakte in vergelijking met de genotype B stam: i) een meer uitgesproken cytoplasmatische actinepolymerisatie onder de bacteriële vasthechtingsplaats, wat resulteerde in een meer efficiënte invasie van de gastheercel, en ii) een vroegere degeneratie van de gastheercel. De genotype D stam kon, in vergelijking met de minder virulente genotype B stam, ook beter overleven en vermeerderen in de kippenmacrofagen. **Deel III A** richtte zich daarom op de studie van het pathogeen karakter van CP3 en 92/1293 *in vivo*, in experimenteel geïnfecteerde (aërosol infectie) specifiek

pathogeen vrije (SPF) kippen. Het was onze bedoeling om de resultaten te vergelijken met deze bekomen tijdens de *in vitro* studie in kippenmacrofagen. Er werd een faryngeale en cloacale kiemexcretie waargenomen bij al de geïnfecteerde dieren. Met behulp van een directe immunofluorescentie kleuring uitgevoerd op vriesweefselsneden van de organen van de geïnfecteerde dieren, kon een systemische infectie worden aangetoond bij alle geïnfecteerde dieren. Stam 92/1293 was ook *in vivo* meer virulent dan CP3 aangezien stam 92/1293 significant ernstigere klinische symptomen veroorzaakte zoals conjunctivitis, rhinitis en ademhalingsproblemen. Stam 92/1293 veroorzaakte, in vergelijking met stam CP3, ook significant ernstigere histopathologische letsels. Dit resulteerde in mortaliteit, welke niet werd gezien in CP3-geïnfecteerde kippen.

In deel B van hoofdstuk III werd de prevalentie van *C. psittaci* in Belgische -en Noord-Franse commerciële vleeskuikenbedrijven onderzocht. Tevens werden de postulaten van Hill-Evans aangetoond voor *C. psittaci* infecties bij vleeskuikens. Dit gebeurde door de infectie experimenteel te reproduceren in SPF kippen, gebruik makend van twee *C. psittaci* genotype B stammen en één *C. psittaci* genotype D stam, die als een aërosol werden toegediend aan de kippen. Deze drie stammen werden geïsoleerd uit braadkippen met een longontsteking. Voor het epidemiologisch onderzoek werden sera van 30 Belgische en 10 Noord-Franse kippenbedrijven onderzocht met behulp van een *C. psittaci* recombinant 'major outer membrane protein' (MOMP) ELISA, die ontwikkeld werd in ons laboratorium. Zesennegentig procent van de onderzochte Belgische vleeskuikenbedrijven was seropositief, terwijl respectievelijk 93% en 90% van de onderzochte Belgische vleeskuikenmoederdierbedrijven en leghenbedrijven seropositief was. Eénennegentig procent van de onderzochte Franse vleeskuikenbedrijven was seropositief. Vervolgens collecteerden we longen van vleeskuikens met pneumonie in een slachthuis. We onderzochten de longen van 5 Belgische en 5 Noord-Franse vleeskuikenbedrijven met behulp van kiemisolatie in celculturen. We konden *C. psittaci* isoleren uit de longen van al de onderzochte Franse bedrijven en uit de longen van 4 van de 5 (80%) onderzochte Belgische vleeskuikenbedrijven. *Chlamydia psittaci* infecties komen bijgevolg vaak voor bij kippen, en dit is in tegenstelling met de gangbare stelling dat kippen minder gevoelig zouden zijn voor *C. psittaci* infecties. Eigenaars van kippenbedrijven en

slachthuispersoneel lopen het risico om geïnfecteerd te worden met *C. psittaci*.

In deel C van hoofdstuk III beschrijven we gedetailleerd de histopathologische letsels die veroorzaakt werden door een experimentele (aërosol) *C. psittaci* infectie van SPF kippen. Hierbij hebben we gebruik gemaakt van de volgende *C. psittaci* stammen: i) 10/423 (genotype B), ii) 10/525 (genotype B) en iii) 10/298 (genotype D). Deze stammen werden geïsoleerd bij Belgische of Franse vleeskuikens met een longontsteking. All stammen veroorzaakten conjunctivitis, rhinitis, sinusitis, tracheïtis, bronchitis, pneumonie, luchtzakontsteking en een ontsteking van de milt, de lever, de nieren en de darmen. De genotype D stam veroorzaakte ook een ontsteking van de eierstokken. De genotype D stam veroorzaakte in het algemeen ernstigere histopathologische letsels en de infectie veroorzaakte hoge mortaliteit (54.5%). De ernst van de letsels nam ook sneller toe bij dieren geïnfecteerd met de genotype D stam, wat suggereert dat deze stam sneller vermenigvuldigde in de organen dan de beide genotype B stammen.

Als besluit kunnen we stellen dat *C. psittaci* een primair pathogeen is voor kippen. Dit wil zeggen dat *C. psittaci* zonder de hulp van andere pathogenen perfect in staat is om een ernstige ademhalingsinfectie te veroorzaken bij kippen. Het voorkomen van *C. psittaci* infecties bij kippen mag met het oog op het dierenwelzijn en het risico voor beroepsgebonden *C. psittaci* infecties bij mensen die werkzaam zijn in de pluimveesector niet langer verwaarloosd worden. Efficiënte monitoring en preventie van deze zoönotische infectie is vereist.

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Curriculum Vitae

PERSONAL DETAILS

Name:	Lizi Yin
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Current Position:	PhD, Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University
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EDUCATION

Ph.D. in Applied Biological Science, Jan. 2010 – present

Faculty of Bioscience Engineering, Ghent University, Gent, Belgium

Dissertation: Epidemiology of *Chlamydiaceae* in livestock and emerging *Chlamydia psittaci* infections in chickens

Promotor: Professor Vanrompay Daisy

M.S. in Veterinary Medicine, Sep. 2007 – Dec. 2009

Faculty of Animal Husbandry and Veterinary Medicine, Jilin University,

Changchun, Jilin Province, China

Dissertation: Studies on a Part of Chemical Constituents and Antibacterial Activity *in vitro* of *Picrorhiza scrophulariiflora* Pennell

Promotor: Professor Xuming Deng

B.S. in Veterinary Medicine, Sep. 2002 – July 2006

Faculty of Animal Medicine, Hunan Agriculture University, Changsha, Hunan Province, China

RESEARCH SKILLS

Molecular biological techniques: PCR, RT-PCR, micro-arrays, gene cloning, protein

expression and purification

Microbiological techniques: culture and isolation of bacteria, handling of hazardous materials and bio-hazardous organisms (*Chlamydiaceae*), screening of pathogenic factor and antigen, pathology in experiment animals

Immunological techniques: ELISA, Immunofluorescence techniques, cytokines detection, immunoblotting

Other techniques: HPLC, gel filtration, silica gel chromatography, nature compounds isolation, NMR, IR, ability working in the biosafety level 3 laboratories, frozen section, animal experiment, develop of veterinary medicine

LANGUAGE

Chinese: native language

English: good

SOFTWARE

Good knowledge of MS Office and Basic software for molecular biology research

SCIENTIFIC PUBLICATIONS

1. **Lizi Yin**[#], Isabelle Kalmar[#], Stefanie Lagae, *et al*. 2013. Emerging *Chlamydia psittaci* infections in the chicken industry and pathology of Belgian and French *Chlamydia psittaci* genotype B and D strains in specific pathogen free chickens. Veterinary Microbiology, 162 (2-4): 740–749. ([#]authors with equal contributions)
2. **Lizi Yin**, Stefanie Lagae, Isabelle Kalmar, Nicole Borel, Andreas Pospischil, Daisy Vanrompay. Pathogenicity of low and highly virulent *Chlamydia psittaci* isolates for specific pathogen free chickens. Avian Diseases. (accepted, 2013)
3. **Lizi Yin**[#], Isabelle Kalmar[#], Jeanne Boden and Daisy Vanrompay. Chlamydial infections in Chinese livestock. OIE Scientific and Technical Review. (accepted, 2013; [#]authors with equal contributions)

Submitted articles

1. **Lizi Yin**[#], Isabelle Kalmar[#], Jeanne Boden and Daisy Vanrompay. Chlamydiosis in Chinese poultry. BMC Veterinary Microbiology. ([#]authors with equal contributions)
2. **Lizi Yin**[#], Isabelle Kalmar[#], Koen Chiers, Isolde Debyser, D. Vanrompay. *Chlamydia psittaci* strains from broiler chickens induce histopathological lesions and mortality in specific pathogen free chickens. Avian Pathology. ([#]authors with equal contributions)
3. **Lizi Yin**[#], Isabelle Kalmar[#], Katelijn Schautteet, *et al*. Prevalence of *Chlamydia abortus*in Belgian ruminants. Vlaams Diergeneeskundig Tijdschrift.([#]authors with equal contributions)

Contributions conferences

1. **Yin Lizi**, Kalmar Isabelle, Schautteet Katelijn, Beterls Guido, Van Driessche Ellen, Czaplicki Guy, Joris Maria-Adelheid, De Zutter Lieven, Borel Nicole, Longbottom David, Frétin David, Dispas Marc, Vanrompay Daisy (2013). Epidemiology of *Chlamydia abortus* in Belgian goat, sheep and cattle herds. 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications (EMAC-2). Jena, Germany, 13-14 June 2013. P37. (abstract)
2. Kalmar Isabelle, Sachse Konrad, Berndt Angela, Chiers Koen, **Yin Lizi**, Vanrompay Daisy (2013). Host-pathogen interactions in specific-pathogen-free chickens following aerogenous infection with *Chlamydia psittaci* and *Chlamydia abortus*. 2nd European Meeting on Animal Chlamydioses and Zoonotic Implications(EMAC-2). Jena, Germany, 13-14 June 2013. P67. (abstract)